INTERACTIONS BETWEEN PLANT GROWTH-PROMOTING RHIZOBACTERIA (PGPR) AND SQUASH PLANTS – THE ROLE OF PGPR-MEDIATED SUPPRESSION OF PHYTOPHTHORA BLIGHT

By

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This dissertation work is dedicated to my husband, Jiebin, for his endless love, support and encouragement during the challenges in research and life. This work is also dedicated to my parents and my baby, who have always loved me unconditionally.
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LIST OF ABBREVIATIONS

ASM   Acibenzolar-S-methyl
BCA   Biological control agent
CFU   Colony forming units
ET    Ethylene
ETI   Effector-triggered immunity
HR    Hypersensitive response
ISR   Induced systemic resistance
JA    Jasmonic acid
LPS   Lipopolysaccharides
MAMP  Microbe associated molecular pattern
PAL   Phenylalanine ammonia lyase
PCR   Polymerase chain reaction
PGPR  Plant growth-promoting rhizobacteria
PO    Peroxidase
PR    Pathogenesis related
PTI   PAMP-triggered immunity
R protein Resistance protein
SA    Salicylic acid
SAR   Systemic acquired resistance
INTERACTIONS BETWEEN PLANT GROWTH-PROMOTING RHIZOBACTERIA (PGPR) AND SQUASH PLANTS – THE ROLE OF PGPR-MEDIATED SUPPRESSION OF PHYTOPHTHORA BLIGHT

By

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Phytophthora blight is a devastating disease in vegetable production worldwide. This disease is caused by the soil-borne Oomycete Phytophthora capsici. The objective of this study was to investigate the suppression of Phytophthora blight on squash and plant defense induced by plant growth-promoting rhizobacteria (PGPR). The biocontrol efficacy of 16 Bacillus PGPR strains to reduce disease severity was evaluated under greenhouse conditions. A rifampicin-resistant mutant of Bacillus PGPR strain IN937b was obtained and used to monitor its colonization on squash roots. The underlying mechanisms of disease suppression by PGPR were determined by analyses of secondary metabolites involving P. capsici inhibition and induced systemic resistance on squash.

In this study, Bacillus PGPR strain IN937b were consistent in reducing Phytophthora blight on squash when applied at the concentration of $10^8$ CFU/mL and on older plants, i.e. the first PGPR application was made at 17 days after emergence. The growth promotion effect of IN937b only occurred under conditions without fertilization. IN937b was capable of producing surfactins and forming biofilms. IN937b colonized the
rhizosphere of squash and also occupied the internal roots. At the early stage of colonization, the population of IN937b remained relatively high in the rhizosphere. The bacterial population in the rhizosphere decreased 14 days after bacterization, while bacterial cells in root tissues survived longer and accumulated gradually. IN937b showed *in vitro* inhibition activities against *P. capsici*. Subtilosin A, an antimicrobial peptide produced by *B. subtilis* IN937b was found to be involved in reduction in zoospore germination, sporangial formation and hyphal growth of *P. capsici*, and the suppression of lesion expansion on the detached leaves after being challenged with *P. capsici*. Greater levels of Peroxidase and phenylalanine ammonia-lyase induced by IN937b may have contributed collectively to induced resistance - a plant innate resistance in squash plants against *P. capsici*, which eventually resulted in lower disease severity compared to the non-treated control. This study provides future researchers with insights into understanding biocontrol mode of action that can be used to develop effective biocontrol agents and to manipulate rhizosphere ecosystems for improved crop production.
CHAPTER 1
LITERATURE REVIEW

Summer Squash

Summer squash (Cucurbita pepo L.), originating from the Americas, is one of widely grown and consumed vegetable crops in the world. As a member of the highly diverse species in the gourd family Cucurbitaceae, is closely related to both muskmelon (Cucumis melo L.) and cucumber (Cucumis sativus L.) (Paris, 2008). The initial domestication of squash occurred in Mexico and Central America about 10,000 years ago, where it spread quickly to Asia, Europe, and Africa, and now it is distributed throughout the world (Smith, 1997).

Summer squash can be used in a wide variety of dishes both as fresh and processing products. The word “squash” comes from a Massachusetts Native American word “askutasquash”, meaning eaten raw or uncooked (Kemble et al., 2005). The fruits, seeds, flowers, shoots and roots of squash are eatable. Consumption of summer squash increases every year (Locke et al., 2009). Summer squash is used as folk medicine because of its nutritional benefits. Since squash contains calcium, magnesium, potassium, vitamins B6 and E, the fruit may contribute to health of the heart, lungs and bones. Squash is also a good source of fiber and protein with a low sugar and sodium concentrations (NASS, 2011).

According to the Food and Agriculture Organization of the United Nations (FAO), more than 1.8 million hectares (ha) of pumpkin, squash, and gourds are harvested annually with a total yield of 24 million tons in 2010 (FAO, 2011). As an easy to grow, short season crop, summer squash is adapted to temperate and subtropical climates and is grown in many regions (Paris, 1996). Today, the largest producers of summer
squash include China, India, Russia, Iran, USA, Egypt and Mexico (NASS, 2012). According to the United States Department of Agriculture (USDA), the United States grew 0.38 million tons of squash for fresh market valued at 248.7 million US dollars in 2012 (NASS, 2013). During 2011-2012, squash growers planted 18,210 ha for fresh market in the United States. The average harvested yield of summer squash is 19.3 tons/ha. The cultivation of squash can be found in a wide latitudinal range from the North (New York) to the South (Florida).

Florida ranks first nationally for the area harvested and value of production on squash processing and fresh market. It is one of the few crops that is shipped every month of the year throughout the United States (Mossler and Nesheim, 2001). The growing acreage was 4,047 ha and the value of the production reached 66 million US dollars in 2012 (NASS, 2012). In 2006, 60% of the squash acreage was in Miami-Dade County (Li et al., 2006). In the Homestead area, where the majority of the state’s squash is produced, squash is grown from August to the following April (Mossler and Nesheim, 2001).

Squash grows best under conditions of high humidity, and temperature between 18 and 24 °C, and properly fertilized soil. Squash plants are largely direct seeded in Florida (Hochmuth and Hanlon, 2010). Sandy loam soil is suitable for squash due to the shallow root system of this crop (Li et al., 2006). The water requirement for squash of approximately one inch of water per week, is slightly lower than that for other vegetable crops requiring (Ertek et al., 2004). Due to hot and dry weather in the winter, more than 90% of squash-producing farms are irrigated to increase production (Mossler and Nesheim, 2001). Under favorable environmental and fertilized conditions, the squash
plants grow rapidly and produce fruits continuously. Squash requires more phosphorus and potassium than nitrogen (Ozoreshampton et al., 1994). Timely and appropriate application of fertilizers can make a significant difference in the quality and quantity of fruits, and may promote early fruit maturation, allowing the fruit to be harvest early.

In Florida, common diseases of squash in the greenhouse and field include pathogens *Fusarium* spp., *Rhizoctonia* spp., *Phytophthora* spp., and *Pythium* spp (Zhang et al., 2007). Diseases cause substantial losses of the yield, up to 20% annually and also decrease the fruit quality (Agrios, 2005). Some diseases occur infrequently such as *Phytophthora* blight (*Phytophthora capsici*), downy mildew (*Pseudoperonospora cubensis*), powdery mildew (*Podosphaera xanthii*), angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*), gummy stem blight (*Mycosphaerella citrullina*) and *Alternaria* leaf spot (*Alternaria cucumerina*) (Zhang et al., 2007). However, when the weather is favorable for disease development, these diseases may cause up to 100% yield loss (Babadoost, 2000).

**Squash Crown and Root Rot Disease Caused by *Phytophthora capsici***

*Phytophthora* blight is a devastating disease of vegetable crops in Florida (Ploetz et al., 2002). This disease is caused by the soil-borne Oomycete *Phytophthora capsici*. *Phytophthora capsici* was first described on chili pepper at New Mexico in 1922 (Leonian, 1922). *Phytophthora capsici* which is widespread throughout the United States has been reported to infect a wide range of plant taxa involving a total of more than 50 species, including major vegetable crops such as cucurbits, peppers, eggplants, tomatoes and snap beans (Granke et al., 2012). In recent years, vegetable producers in eastern U.S. have experienced severe losses of vegetable crops due to *P. capsici* infection (Hausbeck and Lamour, 2004).
Summer squash is highly susceptible to *P. capsici*. Diseased plants are easily pulled from the soil due to the rotted roots system (Padley et al., 2008). Water-soaked lesions on the stem result in girdling and plant death because water and nutrient transport are reduced (Gevens et al., 2008). Affected fruit can be completely covered by sporangia causing fruit to rots, and can occur after harvest during shipment (Ando et al., 2009).

*Phytophthora capsici* releases zoospores from sporangia, or sporangia germinate directly to initiate infection on squash. The zoospores swim through water in the soil and are chemotactically attracted by the exudates released by the host roots (Granke and Hausbeck, 2010). After the zoospores have adhered to the root surface, they encyst and produce a precisely oriented germ tube that grows into adjacent host plant tissue (Raftoyannis and Dick, 2006). The development of zoospores, cysts, and germ tubes is triggered by chemical signals in the environment, some which are produced by the plant roots (Bishop-Hurley et al., 2002).

*Phytophthora capsici* has caused longstanding problems for agriculture. In the field, the disease can spread rapidly in the presence of heavy rainfall and excessive irrigation water. When surface moisture is present, zoospores landing on host plants can invade plant tissues through natural openings such as stomata and hydathodes (gas or water pores, respectively) (Granke and Hausbeck, 2010). The sexual spore of *P. capsici* is the oospore. The oospore’s thick walls help it survive unfavorable periods in the soil. It can also be the overwintering propagule in the soil and the primary source of inoculum (Granke et al., 2009). Dissemination may also occur by contaminated
equipment and through movement of plant debris or other hosts (Lamour and Hausbeck, 2000).

Practices for managing of soil-borne pathogens include crop rotation, fungicide applications, and the use of resistant or tolerant varieties. Field production of vegetable crops in Florida is based on high-input, intensively managed production systems that utilize a broad spectrum fumigants such as methyl bromide to manage soil-borne pests (French-Monar et al., 2007). The fumigant methyl bromide has been used extensively to control soil-borne pathogens, and is effective against the mycelia and the long-term persistent oospores of *P. capsici* in the soil (Zhang et al., 2010). However, agricultural emissions of methyl bromide is a significant cause of ozone depletion (Portmann et al., 2012). Alternatives to methyl bromide either are not as effective as methyl bromide or not economically practical, since squash is not a high value crop (Zhang et al., 2010). With the phase-out of methyl bromide, the ban on this fumigant could potentially cost about 1.5 billion US dollars in lost crop production annually in the U. S. (Sande et al., 2011).

Crop rotation is an important component of integrated disease management; however, the long-term survival of *P. capsici* oospores, even in the absence of a host, limits the effectiveness of this strategy. Crop rotation is needed for more than 3 years to avoid increasing inoculum levels (Hausbeck and Lamour, 2004). In Florida, the oospores survive in the soil up to 5 years. It is very difficult to eliminate the oospores from an infested field (French-Monar et al., 2007).

There are some fungicides registered for use on cucurbits, but none are highly effective against *P. capsici* under hot and humid conditions of South Florida (Mossler
and Nesheim, 2001). Among more than 50 fungicides tested, only six were effective for controlling *P. capsici* (Babadoost et al., 2008). No single fungicide has consistently and effectively suppressed losses caused by *P. capsici*. More importantly, *P. capsici* developed resistance to some fungicides used for Phytophthora blight control. For example, Phytophthora blight often has been managed with application of fungicides containing the active ingredient mefenoxam. Since the mefenoxam-resistant isolates have developed, the usefulness of fungicides containing mefenoxam in the field trials has diminished (Lamour and Hausbeck, 2000).

Plant breeding for resistance is considered an economically viable control method for Phytophthora blight. Commercial cucurbit varieties vary with respect to their Phytophthora blight resistance, but highly resistant varieties with ideal horticultural traits are still not available (Babadoost et al., 2008). *Phytophthora capsici* is a heterothallic species with two mating types, designated as A1 and A2, which interact to produce sexual oospores. The presence of both mating types in the same area provides more potentially virulent phenotypes of the pathogen through sexual recombination. Variation in genetics and virulence among isolates of *P. capsici* lead to costly and time-consuming breeding endeavors (Granke et al., 2012).

At present, there is no single method which can provide adequate control of *P. capsici*. Since the pathogen is difficult to eradicate, induction of plant resistance against pathogen is considered as a potential method in reduction and control of the disease (Shoresh et al., 2010). Plants interact continuously with biotic factors (mycorrhiza, endophytes, insects, nematodes, plant growth-promoting rhizobacteria, and other root-associated microbes) in the field, which might lead to the inhibit the growth of plant
pathogens (Ghorbani et al., 2008), known as biological control or biocontrol. Biocontrol can be developed as a component for sustainably integrated disease management with reduced adverse impact to the environment.

**Plant Growth-Promoting Rhizobacteria**

**Rhizosphere**

The term, “rhizosphere” comes from rhizo or rhiza, a Greek word for root, and “sphere” which means an environment or area influenced by the root (Morgan et al., 2005). In the rhizosphere, plant roots can interact with soil microorganisms, and soil microorganisms can, in turn, affect plant growth and health during the entire growth season (Hartmann et al., 2008).

The rhizosphere is the location where beneficial microorganisms and soil-borne pathogens compete to establish a relationship with the plant (Raaijmakers et al., 2009a). Microorganisms compete with each other to acquire nutrients to grow and multiply (Lugtenberg and Kamilova, 2009). During these processes, metabolic processes of microorganisms result in rapid nutrient cycles and movement of microorganisms in the soil (Raaijmakers et al., 2009a). Some beneficial rhizosphere microorganisms can reduce population densities and activities of soil-borne pathogens by competition, antagonism, and induced plant defense (Weller et al., 2002). Beneficial microorganisms such as plant growth-promoting rhizobacteria (PGPR), positively affect different crops including rice, pepper, tomato, tobacco, and bean (Kloepper and Schroth, 1978). PGPR are a group of free-living rhizospheric bacteria that enhance plant health. PGPR can promote plant health not only directly by facilitating mineral nutrient uptake or phytohormone production, but also indirectly by protecting plants against pathogens (Persello-Cartieaux et al., 2003; Zahir et al., 2004). There have been
many reports that PGPR prevent diseases caused by Oomycetes and fungal pathogens including root diseases (damping-off of tomato), foliar diseases (powdery mildews on cucurbit and strawberry), and postharvest diseases (green, grey and blue molds) (Kloepper et al., 2004).

**PGPR as Biocontrol Agents (BCAs) on Vegetable Crops**

PGPR isolated from different plant species belong to many genera including *Pseudomonas, Rhizobia, and Bacillus* (Lugtenberg and Kamilova, 2009). Multiple *Bacillus* and *Paenibacillus* spp. can be readily cultured from both bulk (free of root) and rhizosphere (influenced by root) soils. The population of PGPR is affected by plant species and root exudates in the rhizosphere. Plant roots release organic compounds such as amino acids and sugars into the rhizosphere (Lugtenberg and Kamilova, 2009), which have a positive effect on the population of many PGPR. The plant rhizosphere is the preferred ecological niche for PGPR. Due to nutrient availability in the rhizosphere, there are about 2.5 to 1,260 times more microorganisms in the rhizosphere than the bulk soil (Rouatt et al., 1960). The population density of *Bacillus* spp. in the rhizosphere generally range from $10^3$ to $10^7$ cells per gram of rhizosphere soil (Pandey and Palni, 1997).

PGPR are effective in controlling plant diseases caused either by soil-borne, foliar or post-harvest pathogens (Raupach and Kloepper, 1998b; Kloepper et al., 2004). PGPR were isolated from different crops (tomato, pepper, cucumber, etc.) based on their multiple plant growth promoting and antifungal capabilities. These PGPR isolates are well adapted when they are introduced to particular rhizospheric soil environment (vanVeen et al., 1997). The target pathogens include soil-borne fungal pathogens including *Phytophthora, Fusarium*, and *Rhizoctonia*, as well as foliar diseases caused
by bacterial pathogens Xanthomonas and Ralstonia. Several commercial PGPR products are currently available for use on vegetables and new products are constantly being developed and commercialized in the market (Kumar et al., 2011b). The details of commonly used commercial PGPR products for managing important diseases of vegetables are listed in Table 1-1.

PGPR as biocontrol agents have the following advantages: (1) PGPR which were isolated from the rhizosphere or plants are environmentally friendly and nontoxic (Beneduzi et al., 2012), (2) PGPR utilize root exudates leading to their rapid growth and mass production (Lugtenberg and Kamilova, 2009), (3) PGPR adapt to the environment by colonizing and multiplying in the rhizosphere and interior of the plant (Compant et al., 2010), and (4) PGPR undertake a defined range of functions including antibiosis, growth promotion, and induced systemic resistance (Compant et al., 2005).

The success of biocontrol agents mainly depends on the existing seed, soil, or foliar application technologies that help integrate PGPR into the cropping system. Seed inoculation with the P. fluorescens isolate CW2 positively affects tomato growth and protects seedlings from damping-off disease caused by Pythium ultimum (Salman and Abuamsha, 2012). A foliar spray of P. fluorescens Pf1, Py15, and B. subtilis Bs16 significantly reduced the incidence of early blight caused by Alternaria solani (Latha et al., 2009). The most commonly used application method for PGPR is as a soil drench. Soil application of Bacillus subtilis QST713 consistently reduced infection by Plasmodiophora brassicae on canola roots. Antibiosis and induced resistance are involved in clubroot suppression by QST713 (Lahlali et al., 2013). The PGPR strains
aggressively colonized plant roots, thus helping to inhibit pathogens in the rhizosphere by producing antibiotics and inducing host resistance.

The dosage of the BCAs significantly influence the biocontrol efficacy through their effect on establishment of pathogens and the development of diseases. To achieve a beneficial effect on a target plant, PGPR have to be implemented into the field in a timely manner and in high numbers. In the field, a cell concentration at $10^9$ CFU per mL of *Pseudomonas brassicacearum* MA250 was required for effective biocontrol of *Fusarium* spp. on winter cereals (Levenfors et al., 2008). The implemented PGPR strain had to reach a threshold population density in order to effectively suppress pathogens in the rhizosphere. For some PGPR strains, the maximum root colonization was less than $10^6$ CFU per gram of root in the nutrient-rich environment (Ahmad et al., 2011). *Pseudomonas putida* strain WCS358 reached a threshold population density of approximately $10^5$ CFU per gram of root, which was required for a significant suppression of Fusarium wilt on radish (Raaijmakers et al., 1995b).

Host age influences the effectiveness of PGPR against pathogens. Plants vary in their susceptibility to certain pathogens due to their age or stage of development, known as age-related resistance (Garcia-Ruiz and Murphy, 2001). In general, young plants are susceptible to pathogens while adult plants have a certain degree of resistance. Age-related resistance to *P. capsici* is found in cucumber. Immature fruits are more susceptible to *P. capsici* than mature fruits (Meyer and Hausbeck, 2013). Plant age also affects PGPR populations through a modification in the root exudation (Roesti et al., 2006). At a specific age of the host plant and the appropriate timing of PGPR application, a plant may respond to PGPR and pathogens by activating its resistance.
locally and systemically (Katagiri and Tsuda, 2010b). Some proteins and metabolites form during this particular period. A resistance protein (R protein) - mediated recognition of *P. syringae* is involved in age-related resistance in *Arabidopsis* (Kus et al., 2002). Salicylic acid (SA) signaling was involved in age-related resistance of *Nicotiana benthamiana* against *P. infestans* (Shibata et al., 2010). Young and susceptible plants failed to induce the salicylic acid (SA) - mediated signaling pathway. Pretreating young plants with acibenzolar-S-methyl (ASM, Actigard®, Syngenta Crop Protection, Greensboro, NC, USA) or SA analogs induced resistance to *P. infestans*.

**Root Colonization of PGPR**

The first step in all interactions between plants and PGPR is bacterial colonization of plant root systems. The steps of colonization include recognition, adherence, invasion, occupation and multiplication, and signals to establish the interactions (Compant et al., 2010). Effective root colonization by PGPR depends on their ability to survive and proliferate in the presence of indigenous microorganisms over a period of time (Maheshwari, 2011). For example, *P. fluorescens* 2112 consistently lasts for 18 weeks in the rhizosphere of pea (Kim et al., 2012).

Chemotaxis is one way that plants attract PGPR to the rhizosphere through the release of carbohydrates and amino acids (Somers et al., 2004). The major chemo-attractants in tomato root exudates for *P. fluorescens* WCS365 were identified as leucine and dicarboxylic acids (Lugtenberg et al., 1999). Another organic acid, malate, appeared to be the major chemo-attractant for *B. subtilis* FB17 in root exudates of *Arabidopsis thaliana* (Rudrappa et al., 2008). Biocontrol agents may be distributed in the specific crop or the different niches of the hosts (Beneduzi et al., 2008). For example, *B. amyloliquefaciens* FZB42 primarily colonized in root hair and tips on
Arabidopsis. However, when FZB42 was applied to maize roots, the bacterial cells concentrated at the base of primary roots indicating that FZB42 colonized plant roots in different niches (Fan et al., 2011).

Some bacteria present in the rhizosphere are capable of entering the plants as endophytes that do not cause harm and can establish a mutualistic association with plants. Endophytic bacteria have been isolated from a wide range of plant species. In general, endophytic bacteria occur at lower population densities than rhizospheric bacteria (Compant et al., 2010). The population density of endophytes is greatly variable, depending mainly on the bacterial species and genotypes of the host plant, also influences by the developmental stage of the host, inoculum density, and environmental conditions (Sturz et al., 2005).

Populations of PGPR usually develop and form biofilms in their natural environments (Seneviratne et al., 2010). Biofilms are a group of highly structured cells capable of sticking together on the surface of liquid or solid substances in a self-produced extracellular matrix (Costerton, 1995). Biofilms change the growth rate of cells and transform from free-living cell to sessile biofilm cells through nutrient limitation (Morikawa, 2006). Biofilm formation is important for PGPR to act as a biocontrol agent against plant pathogens and to respond quickly to environmental conditions (Seneviratne et al., 2010). Paenibacillus polymyxa protected Arabidopsis thaliana against Phytophthora palmivora and Pythium aphanidermatum by establishing biofilm on plant roots to protect the colonization sites such as root tips. Competition for nutritional elements in the root exudate, biofilm-forming PGPR act as a sink for the nutrients can inhibit pathogen growth in the rhizosphere (Timmusk et al., 2005). In
addition, biofilm-forming PGPR can produce a variety of antimicrobial metabolites which include broad spectrum lipopeptides such as surfactins that are potent biosurfactants for maintaining structure of biofilms and are important for suppressing pathogens (Bais et al., 2004b).

Although beneficial aspects of PGPR have been well documented, there are some problems about variability in colonization efficiency, field performance, and rhizosphere competence (Babalola, 2010). In the field, PGPR behave unpredictably in different environments (Spadaro and Gullino, 2005). Variability in rhizosphere colonization has been proposed to be a major obstacle to agronomic application of promising PGPR strains (Cooper and Rao, 2006). Research into the mechanisms of action has provided a comprehensive understanding of PGPR as biocontrol agents. Research on the mechanism that is responsible for how the PGPR interact with the hosts and pathogens would help to improve the efficacy of growth promotion and biocontrol. Detailed studies of the whole system need to be undertaken in order to reduce the variability of PGPR performance (Compant et al., 2005).

**Mode of Action of Biocontrol Agents**

The process of biocontrol is the action of suppressing pathogens by using PGPR with a variety of mechanisms such as antagonism mediated by metabolites, competition for nutrients and niches in the rhizosphere, and/or activation of plant defenses. Most of successful commercial BCAs are based on the thorough knowledge of their modes of action, not only under laboratory conditions, but also in greenhouse and field situations.

**Antagonism Mediated by Metabolites**

Antagonism is usually mediated by the production of secondary antimicrobial metabolites (antibiosis), lytic enzymes, and/or effectors (Raaijmakers et al., 2009a). In
the case of PGPR, antibiotics are secondary metabolites with low-molecular weight that are toxic to other microorganisms (Mannanov and Sattarova, 2001). The metabolites released by PGPR are involved in directly inhibiting plant pathogens locally before they reach the plant (Kessler, 2011). Only a small fraction of pathogen populations is exposed to the antimicrobial compounds in the rhizosphere during a short period of its life cycle (Raaijmakers et al., 2009b). Therefore, resistance in pathogens to antimicrobial compounds produced by antagonistic PGPR is presumed not to develop, or at least develops relatively slowly compared to resistance to chemicals in pathogen populations (Ishii, 2006).

*Pseudomonas* and *Bacillus* species are known to produce antibiotics that are inhibitory to the growth and/or activities of fungal pathogens of plants. Well-known and characterized compounds produced by *Pseudomonas* are phenazine-1-carboxylic acid (PCA) and 2, 4-diacylphloroglucinol (DAPG), while cyclic lipopeptides surfactants, zwittermycin A, and bacteriocins can be produced by *Bacillus* (Hayat et al., 2010). To demonstrate the role of antibiotics in biocontrol, the mutants defective in structural genes for synthesis of the antibiotic must be negative in biocontrol. In a previous study, knock-out mutants of lipopeptides surfactin genes were severely defective in biofilm formation *in vitro* and in colonization of tomato roots. At the same time, the mutant decreased the efficacy of biocontrol against *Ralstonia solanacearum* on tomato under greenhouse conditions (Chen et al., 2013). DAPG is a primary factor contributing to biological control of plant disease by *P. fluorescens* strains CHA0 and several *Pseudomonas* strains. The 2, 4-DAPG biosynthetic and regulatory mutants demonstrated that DAPG has a broad spectrum of antifungal activities that inhibit
pathogens directly, and also induce systemic resistance in the host plants (Weller et al., 2012).

**Competition for Nutrient and Niches**

An introduced PGPR strain can be defined as an effective root colonizer if it is able to propagate and survive in the rhizosphere for several weeks. PGPR face the challenge of carbon starvation in most soils (Knee et al., 2001). Competition for different carbon sources generally occurs in the soil between the introduced PGPR strains and the native inhabitants in the rhizosphere (Nihorimbere et al., 2011). For this purpose, PGPR directly inhibited pathogens under highly competitive conditions (Cunniffe and Gilligan, 2011). For example, the lack of carbon sources is assumed to inhibit fungal spore germination in the soil providing a broad spectrum of protection on plants (Tang et al., 2010).

PGPR can compete with pathogens for niches when they colonize the same host tissues and develop in different root cortical cells, indicating some sort of competition for space (Ongena and Jacques, 2008). A number of bacterial functions such as motility, attachment, growth, stress resistance and the production of secondary metabolites have been associated with competence for space occupation in the rhizosphere (Lugtenberg and Kamilova, 2009).

**Defense Mechanisms in Plants**

Plants possess morphological barriers, secondary metabolites, and antimicrobial proteins that prevent or overcome a pathogen’s ability invading a plant (van Loon et al., 2006). Plants possess different strategies to recognize and counteract pathogen attacks. As a first line of defense, the plant cell surface contains pattern recognition receptors (PRRs) that recognize potential pathogens through flagella, outer membrane
lipopolysaccharides (LPS), and other cell wall or secreted components (Zipfel, 2008). In addition to flagella and LPS, there are various bacterial compounds that can be recognized by the plant, including N-acyl-homoserine lactones, biosurfactants, siderophores, and the antibiotics DAPG and pyocyanin (Doornbos et al., 2012).

General elicitors that are designated as pathogen-associated molecular patterns (PAMPs) are isolated from pathogens (Boller and He, 2009). Non-pathogenic microorganism elicitors are designated as one of microbe-associated molecular patterns (MAMPs), which are recognized in a similar way (Bittel and Robatzek, 2007). The perception of general elicitors triggers a broad array of reactions, known as basal resistance or PAMP-Triggered Immunity (PTI). However, some successful pathogenic microorganisms may overcome basal resistance by inhibiting signaling pathways, thus suppressing this first type of immunity (Zamioudis and Pieterse, 2012). In response, plants have evolved a second line of defense through specific disease resistance (R) genes, the so-called effector triggered immunity (ETI) (Katagiri and Tsuda, 2010a). R gene-mediated resistance is expressed through similar defense responses as those that are active in basal resistance, but on a much greater scale (Henry et al., 2012). Therefore, PTI and ETI are considered as primary and secondary innate immunity, respectively.

The phenomenon of induced resistance to pathogens in plants has been studied intensively in recent years (Hammerschmidt, 1999). Induced resistance can be divided into systemic acquired resistance (SAR) and induced systemic resistance (ISR). In SAR, resistance can be activated by chemicals, avirulent pathogens, or attenuated pathogens. In contrast to SAR, ISR is induced in roots by PGPR. During ISR, gene
expression is induced locally in roots or systemically in leaves upon colonization by PGPR, and the treated plants are in primed conditions (i.e. defense genes respond more rapidly and strongly after pathogen attack).

**ISR Mediated by PGPR**

To fully understand the specific mechanism of ISR, research on ISR in certain crop species mediated by specific PGPR strains is required. Globally, local and systemic defense responses triggered by microorganisms are controlled by a signaling network in which the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play important roles (Henry et al., 2012).

Induction of SA-mediated responses has been demonstrated to occur in the rhizosphere (Doornbos et al., 2012). Several phytohormones have been demonstrated to negatively cross-communicate with the SA signaling pathway and affect the outcome of the immune response (Verhagen et al., 2004; Pieterse et al., 2009). Local JA and ET signaling play a role in the control of beneficial interactions (Lopez-Raez et al., 2010). ET also appears to have a crucial role in regulating the progress of root colonization (Choudhary and Johri, 2009). For example, a significant reduction in the expression of genes encoding ET-related transcription factors has been reported for *Arabidopsis* roots colonized by *P. fluorescens* WCS417 and FPT9601-T5, which supports the hypothesis that PGPR modulate host immune response by interfering with the ET signaling pathway (Verhagen et al., 2004).

Most pathogenicity-related (PR) proteins are induced through the action of the signaling compounds and possess antimicrobial activities (van Loon et al., 2006). *B. cereus* strain BS107 against *Xanthomonas axonopodis pv. vesicatoria* systemically induced plant defense genes *CaPR1* and PR4 in pepper leaves (Yang et al., 2009). In
tobacco (*Nicotiana tabacum* cv. Samsun NN), at least 16 PR-1-type genes appear to be present after being induced upon TMV infection (Riviere et al., 2008). These findings indicate that PR proteins classified in the same family on the basis of sequence homology can have different properties and hence may differ substantially in biological activity. Examples of PR proteins include phenylalanine ammonia-lyase (PAL) (Mariutto et al., 2011), peroxidase (Sarwar et al., 2011), and polyphenoloxidase (Mayer, 2006). They are generally present constitutively and only increased the expression level during most infections.

Plants recognize certain ISR determinants to elicit ISR. Volatile organic compounds and more particularly 2,3-butendiol were the determinants for elicitation identified from *Bacillus* spp. (Ryu et al., 2003). Massetolide A produced by *P. fluorescens* retains ISR-eliciting activity in tomato plants for the control of *Phytophthora infestans*, the causal agent of late blight (Tran et al., 2007). The compound 2,4-diacetylphloroglucinol (DAPG) may also act as an elicitor of systemic resistance in some *Pseudomonas* strains (Bakker et al., 2003). Another class of compounds emerged recently as ISR elicitors is biosurfactant such as rhamnolipids and lipopeptides. Pure fengycins and surfactins from *B. subtilis* provided a significant protective effect that is similar to the one induced by living cells of the producing strain. Experiments conducted on bean and tomato showed that overexpression of both surfactin and fengycin biosynthetic genes in *B. subtilis* strain 168 (the strain is impaired in lipopeptide production) was associated with a significant increase in the potential of the derivatives to induce resistance (Ongena et al., 2007).
B. subtilis mutant strain M1 was constructed with a deletion in a surfactin synthase gene and thus deficient in surfactin production (Bais et al., 2004b). Bacillus subtilis M1 was ineffective as a biocontrol agent against P. syringae in Arabidopsis and also failed to form robust biofilms on either roots or inert surfaces (Bais et al., 2004b). The understanding of the physiological and biological basis of these induced immunity mechanisms has greatly advanced the development of novel strategies for disease control (Henry et al., 2012). The importance of these considerations is expected to have practical applications. PGPR have the ability to control pathogens through complex processes of colonization and antibiotic production. Studies of the population dynamics and physiology of PGPR can be important and useful for applying PGPR for disease management in vegetable production.

The aim of this dissertation was to investigate PGPR strains for controlling Phytophthora blight on squash. The first objective was to determine the effectiveness of Bacillus PGPR strains in the biocontrol of crown and root rot disease on squash caused by P. capsici in soilless potting mixes under the greenhouse conditions. The second objective was to focus on understanding of the ecology of Bacillus PGPR and pathogen to enhance the effective use of Bacillus PGPR in the suppression of plant diseases. The third objective was to gain insights into the role of antifungal antibiotics in disease suppression via antagonism. The fourth objective was to gain an understanding of mechanism in induced resistance by monitoring the defense-related enzyme activities changes on squash to impair pathogen progression locally and systemically.
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<th>Target pathogen</th>
<th>Crop</th>
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<td><em>Colletotrichum orbiculare</em></td>
<td>Cucumber</td>
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CHAPTER 2
BIOLOGICAL MANAGEMENT OF PHYTOPHTHORA BLIGHT AND PLANT GROWTH PROMOTION BY SELECTED PGPR STRAINS UNDER GREENHOUSE CONDITIONS

Introduction

Squash (Cucurbita pepo L.), in the family Cucurbitaceae, is a widely grown and consumed vegetable crop in the United States and many other countries as well. In 2012, growers in the United States harvested 340,060 tons of squash for fresh market valued at $248.7 million (NASS, 2011). Ranked No. 1 nationally in terms of the acreage harvested and the value of processing and fresh market production, squash became a valuable vegetable crop for Florida growers. Squash is vulnerable to bacterial and fungal pathogens. The most devastating squash disease is Phytophthora blight (Babadoost, 2000), which can cause yield losses up to 100% in warm and humid conditions (Hausbeck and Lamour, 2004). Phytophthora blight poses major challenges to growers by reducing yield and adversely affecting the fruit quality.

Phytophthora blight is a soil-borne disease which commonly affects numerous crops in the United States including all cucurbits, peppers, tomatoes, snap beans and several weed species (Ploetz and Haynes, 2000). The pathogen that causes this disease is the Oomycete Phytophthora capsici Leonian (Leonian, 1922), which produces motile zoospores. When it rains, or irrigation water splashes in the field, zoospores can be spread with water to reach healthy squash tissues (Granke et al., 2012). Zoospores and sporangia can infect all parts of squash plants including leaves, roots, stems and fruits (Hausbeck and Lamour, 2004). The affected plants quickly die in a week due to break down of the root and/or stem systems.

Symptoms of Phytophthora blight are first visible as water-soaking lesions, which then turn dark over time (Kousik et al., 2012). Phytophthora capsici is easy to identify on
the fruit or stem as white mass of mycelia, sporangiophores and sporangia on the surface of the tissue (Roberts et al., 2005). This diploid Oomycete is heterothallic with two different mating types to complete sexual cycle (Erwin and Ribeiro, 1996). When both mating types A1 and A2 are found in the same field, the sexual propagule oospores are produced. Oospores can tolerate adverse environments and survive in the soil for up to five years (French-Monar et al., 2007). In the spring, oospores are the primary source for initial infection in the field (Pavon et al., 2008). Therefore, controlling this disease has become increasingly challenging because oospores can survive a long time in the soil, even without host tissues. Oospores have the ability to infect all parts of plants, and more aggressive types of this pathogen may potentially cause frequent outbreaks (Sanogo and Ji, 2012). So far, there is no single strategy that provides adequate protection against this disease.

In an effort to prevent or control Phytophthora blight, resistant cultivars remain the most economical and long-term approach. Although genetic resistance was identified from some cucurbit species, there is no cucurbit cultivar commercially available with measurable resistance to *P. capsici* (Hausbeck and Lamour, 2004). Mapping genes for resistance to Phytophthora blight is challenging, and breeding for resistant cucurbits is complex due to continuous changes in the genetics of *P. capsici* (Babadoost et al., 2008).

In Florida, intensively managed production systems for vegetable production rely on methyl bromide, a fumigant commonly used to manage soil-borne pests (French-Monar et al., 2007). With the impending phase-out of methyl bromide (Noling and Becker, 1994), growers must apply fungicides frequently to maintain their productivity.
However, overusing chemicals increases the risk of the pathogens developing resistance to fungicides, resulting in the reduction of chemical efficacy. Chemicals adversely impact non-target species and the environment, causing potential public health concerns (Pimentel et al., 1992). As a result, there is an increasing demand for the development of biologically based, environmentally safe alternatives to chemical pest-control products.

The use of microorganisms to control plant disease is a promising strategy and has potential for improving plant health (Cook, 1993). As an eco-friendly and cost-effective strategy, biological control agents (BCAs) are usually isolated from natural fields or the inside of plant tissues (Zhang et al., 2009). These BCAs are capable of colonizing the root surface efficiently when they are reintroduced into the soil. Once established, BCAs are capable of providing long-lasting and long-term defenses against soil-borne pathogens attack.

Many members of the genus Bacillus have the ability to effectively control plant diseases. Bacillus subtilis is an ideal example of a bacterial biological control agent, which is able to control damping-off caused by Pythium ultimum (Asaka and Shoda, 1996), crown rot caused by Aspergillus niger (Podile and Prakash, 1996), and vascular wilt caused by Fusarium oxysporum (El-Hassan and Gowen, 2006). Bacillus subtilis has also been developed commercially for bio-control products including BioYield® (Kloepper et al., 2007), Subtilex® (Schisler et al., 2004) and Serenade® (Lahlali et al., 2013).

Bacillus strains have several characteristics making them good candidates for developing competitive BCAs. First, Bacillus spp. have the potential to produce more
than 20 antibiotics with strong antimicrobial activity (Stein, 2005). Antibiotics help 
_Bacillus_ strains successfully survive and multiply in the soil and to compete with other 
microorganisms for nutrients and space (Nihorimbere et al., 2011). _Bacillus_ PGPR have 
been shown to increase seed emergence, plant weight, and yield (Kloepper et al., 
2004). _Bacillus_ spp. secrete plant hormones such as indole-3-acetic acid (IAA), 
cytokinin and gibberellin which enhance plant growth (Spaepen et al., 2007). _Bacillus_ 
strains also increase plant uptake of available minerals, nitrogen, and phosphorus from 
the soil (Toro et al., 1998). _Bacillus_ strains form heat- and desiccation- resistant 
endospores that promote survival in stressful environments (Lim and Kim, 2010).

Commercialization and the use of most bio-control products remains limited due
to inconsistency in their efficacy, particularly under field conditions (Ramaekers et al.,
2010). To exert their beneficial effects, the application method and timing is critical for
avoiding an outbreak of the pathogen prior to the introduction of _Bacillus_ spp. into the
soil (Spadaro and Gullino, 2005). To solve this problem, the appropriate methods and
timing for applying BCAs need to be determined. Application timing is important
because BCAs need to be applied prior to a pathogen establishing itself on a host plant
in order to achieve the best control. Host may regulate its defense response to a
pathogen during its different growth stage, known as age-related resistance (Kus et al.,
2002). Appling BCAs to a plant host prior to the plant’s maturation is imperative to
impede the contact of the pathogen with the host plant. A positive correlation has been
shown between a plant age and resistance to _Phytophthora megasperma var sojae_ in
soybean(Hanley et al., 1995). Thus, the application timing of BCAs may directly
influenced by age-related resistance.
It is generally assumed that the biocontrol efficiency and yield are higher when BCAs are used in a high dosage. Application rate can be altered to maximize BCAs efficacy. However, it is probable that introducing too many of microbes could negatively affect the plant or the rhizosphere (Strigul and Kravchenko, 2006). For example, population growth of PGPR is suppressed when PGPR population density is high (over $10^9$ CFU/g soil) due to competitiveness for resources and production of secondary metabolites. Additional work is needed to determine the optimal application rate of BCAs in for efficient control.

In previous research, PGPR strains from Auburn University (Dr. Joseph Kloepper’s lab) isolated originally from the plant rhizosphere were efficient in plant growth promotion and disease control against multiple pathogens on different vegetable crops under greenhouse and field conditions (Zhang et al., 2010). The overall goal of the present study was to evaluate bio-control agents including PGPR for suppressing the Phytophthora blight on squash. The specific objectives were to (1) investigate the potential of 
* Bacillus * spp. for bio-control of Phytophthora blight on squash; (2) optimize the application rate and timing for better control of this disease.

**Materials and Methods**

**PGPR Strains and Their Growth Conditions**

Sixteen bacilli PGPR strains provided by Dr. Joseph W. Kloepper at Auburn University, AL were isolated from the soil rhizosphere, root surfaces, and roots of different crops. Information on these PGPR strains are listed in the Table 2-1. PGPR strains used in this study were stored in Nutrient Broth (NB, pH=7.0) containing 20% glycerol at -80 °C.
PGPR strains were streaked on nutrient agar (NA) and incubated at 28 °C for 24 h. Single colonies were inoculated in NB and cultured for 24 h in a shaker incubator (SteadyShake 757, Amerex Instruments, Inc., Lafayette, USA) at 150 revolutions per minute (rpm) at 28 °C. The bacterial culture was centrifuged at 3,000 rpm for 10 minutes (min) and the cells were suspended in 20 milliliter (mL) of sterilized deionized water (DI water). The concentrations of the bacterial suspensions were adjusted to 10⁸ cells/mL based on the optical density (OD₆₀₀) with a spectrophotometer (UNICO SpectroQuest TM 4802 UV/VIS Double Beam, United Products & Instruments Inc., NJ).

**Media Used in the This Study**

Pimaricin–ampicillin–rifampicin–pentachloronitrobenzene with hymexazol cornmeal agar (PARPH) medium was modified (Mitchell and Kannwischer-Mitchell, 1992): corn meal agar (Difco, 17 g/L) amended with: ampicillin (100 mg/L), hymexazol (50 mg/L), pimaricin (10 mg/L), PCNB (100 mg/L), rifampicin (10 mg/L).

Clarified V8 broth was prepared with 250 mL V8 juice by mixing with 0.2 g CaCO₃ and centrifuging at 3,000 rpm for 20 min at room temperature. The supernatant was filtered through Whatman® No. 1 filter paper. The final volume of supernatant was 200 mL and mixed with 800 mL DI water.

V8 agar (unclarified) was prepared with 10% V8 juice, 1% CaCO₃ and 15 g/L Agar. The mixture was heated and stirred prior to autoclaving in order to dissolve the solutes completely.

**Growth of Squash Plants**

Zucchini (*Cucurbita pepo* cv. Senator) seeds were obtained from S&B Inc. (Homestead, FL). The seeds were washed in DI water (pH 2.0, adjusted with 6M HCl) to remove fungicides in the coating, and grown in the plastic pots (7.62 cm high and 10.16
cm at the opening with 8 drain holes) containing potting mix (Fafard® Lightweight Mix 2, Conrad Fafard, Inc., Aquawam, MA) in the greenhouse (24 ± 10 °C) during the experiments. After the squash plants had two completely expanded leaves, each seedling was fertilized with 50 mL (3.5g/L) of a liquid soluble fertilizer (Peter’s 20:20:20 NPK, Peters Fertilizer Products, Fogelsville, PA) every two weeks.

**Application of PGPR Treatments in the Greenhouse**

To evaluate the effect of PGPR on Phytophthora blight and plant growth, PGPR were applied two times in the greenhouse. For the first PGPR application, 20 mL of each PGPR suspension at $1 \times 10^8$ CFU/mL was applied as a soil drench around the base of the stem. One week later, the plants were treated with the same PGPR a second time by soil drench to enhance the PGPR population and potential of disease control.

**Inoculum Preparation and Inoculation**

Isolates (#121, #146, and #151) of *P. capsici* were provided by Dr. Pamela D. Roberts, Southwest Florida Research and Education Center, University of Florida, Immokalee, FL. The inoculum of *P. capsici* was prepared according to (Ploetz et al., 2002) with slight modifications. *Phytophthora capsici* was grown on the selective medium, PARPH, containing a mixture of fungicide and antibiotics to prevent contamination by bacteria and other soil-borne pathogens. A disc (5-mm diameter) was cut from the edge of the medium with *P. capsici*, transferred to V8 agar and grown for one week at 25 °C. Ten discs (5 mm) with *P. capsici* were cut from the V8 agar plate, transferred to a Petri dish (90-mm diameter) containing 20 mL of V8 broth and incubated for one week at 25 °C in the dark. The mycelium in the V8 broth was washed twice with DI water, and 15 mL of DI water was added to merge the mycelium. Then the
plates were placed under light at room temperature for 2 days to induce sporangial formation. The plates were chilled at 4 °C for 1 hour, before they were moved into an incubator at 28 °C for 30 minutes to enhance the release of zoospores. The concentration of zoospores was determined under an Olympus BH2-RFCA microscope (Olympus, Tokyo, Japan) using a hemocytometer after the sporangial solution was stirred in a vortex mixer for 1 min to break flagella of the zoospores.

For inoculation with *P. capsici*, the concentration of the inoculum was adjusted to 2×10^4 zoospores/mL using DI water. Three days after the second PGPR treatment, 5 mL of the zoospore inoculum was carefully added to the soil around the base of plant stems. Inoculated plants were maintained on benches in the greenhouse with high soil moisture by hand watering daily.

**Disease Rating**

Symptoms of Phytophthora blight usually started at the base of the stem about 4-5 days after inoculation. The disease was rated according to a 0-5 scale (Zhang et al., 2010), where 0=healthy plant, 1= water soaked lesions at the bottom of the stem, 2= stem was girdled and had lesions or started to exhibit white mycelium on the surface of diseased tissue, 3= all leaves turned yellow and the whole plant started to collapse, 4= plants wilted and fell down, and 5= dead plant.

**Evaluation of PGPR for Suppressing Phytophthora blight**

Sixteen PGPR strains were tested in two groups. Each group included eight PGPR strains, one standard fungicide (Presidio®) and a non-treated control. There were ten plants in each treatment with each plant as a replicate. The experiment was designed as a randomized complete block. Each experiment was conducted three
times. The replications and duration of experiments were similar in all trials unless specified.

**Plant Growth Promotion by PGPR**

To evaluate the effect of PGPR on plant growth promotion, soil was drenched with PGPR suspensions ($1 \times 10^8$ CFU/mL) twice after two plant leaves were completely expanded. In order to test the influence of fertilizers on plant growth promotion by PGPR treatment, each pot received 20 mL (3.5 g/L) of the liquid fertilizer 20:20:20 NPK mixture (Peters General Purpose 20-20-20; Peters Fertilizer Products, Fogelsville, PA) 3 days after the first PGPR application. In another experiment, plants were grown in the potting mix without fertilization. Ten days after the second PGPR treatment, plant height, stem diameter, fresh and dry weight of shoot and root, root length were measured and recorded. The experiment was designed as a randomized complete block, and ten plants were used in each treatment with each plant as a replicate.

**Effect of PGPR Concentration on Disease Suppression**

The effect of PGPR concentration on disease control efficacy was determined by soil drench with PGPR suspensions at $10^5$, $10^6$, $10^7$ and $10^8$ CFU/mL. The different PGPR concentrations were prepared by serial dilution from $10^8$ CFU/mL with DI water. The standard plant resistance inducer Actigard® was included in this experiment. Each pot was treated with 10 mL of Actigard (30 mg/L) at the same time that the PGPR treatment was applied.

**Effect of Timing of PGPR Application on Disease Suppression**

The effect of timing of PGPR application on Phytophthora blight in squash was evaluated at different plant growth stages. The first PGPR application was applied 10 (Stage 1), 17 (Stage 2) and 24 (Stage 3) days after emergence in each individual trial.
The second PGPR application was applied 7 days after the first application.

*Phytophthora capsici* inoculation was the same as described previously.

**Effect of PGPR on Chlorophyll Content in Squash Leaves**

The chlorophyll content in squash leaves was estimated with a Minolta SPAD 502 meter (Minolta Camera Co., Osaka, Japan) to determine the effect of PGPR treatment on chlorophyll contents. The data were collected one day before inoculation, and five days after inoculation when plants started to show symptoms. The readings were taken from the 3rd, 4th, 5th leaf from apex in all treatments. Data were averaged from three different leaves for each plants.

**Statistical Analyses**

Data of disease severity was collected using the rating system from 0 (no disease) to 5 (dead plant). The area under the disease progress curve (AUDPC) was calculated for each replicate plant in each treatment using the formula

\[ \sum_{i}^{n-1} \left[ \frac{y_{i} + y_{i+1}}{2} \right] (t_{i+1} - t_{i}), \]

where \( y_{i} = \) disease severity at the \( i \)th day, \( t_{i} = \) time at the \( i \)th day, and \( n = \) total number of evaluation times (Ristaino, 1991).

All analyses were performed by using the statistical analysis system (SAS version 9.1, SAS Institute, Cary, NC). Data of AUDPC and growth variables in response to different PGPR strains in the greenhouse tests were analyzed by a one-way analysis of variance (ANOVA). Significant differences among means were determined with Fisher’s protected LSD test \( (p = 0.05) \).
Results

Susceptibility of Squash to Different *P. capsici* Isolates

Isolate 121 was the most aggressive among three *P. capsici* isolates (121, 146, and 151) tested, resulting in highest disease severity and AUDPC value at 10³, 10⁴ and 10⁵ zoospores/mL 8 days after inoculation (Table 2-2). Isolate 146 was had a slight effect on inducing disease symptoms at 10⁵ zoospores/mL, and isolate 151 had a moderate effect based on the fact that Phytophthora symptoms were the lowest at 10⁴ and 10⁵ zoospores/mL. Based on the result, isolate 151 was chosen to use in the following experiments. The isolate 151 at 1x 10⁵ zoospores/mL incited significantly greater disease than 10⁴ zoospores/mL, indicated as AUDPC values (Table 2-3). There was no significant difference in AUDPC values by *P. capsici* from 2x10⁴ to 8x10⁴ zoospores/mL. Next, we analyzed the correlation between zoospores levels and disease development, there would have a positive relationship between zoospores concentrations and AUDPC values (Figure 2-1). Based on the results above, 2x10⁴ zoospores/mL of isolate 151 was selected as the *P. capsici* inoculum concentration for testing the effect of PGPR on suppression of Phytophthora crown and root rot, unless indicated otherwise.

Effect of PGPR on Phytophthora Blight in the Greenhouse

Sixteen selected *Bacillus* strains of PGPR were evaluated in the greenhouse for suppressing Phytophthora blight - in this case, Phytophthora crown and root rot. In the progression of disease development, some PGPR strains significantly (*P*≤0.05) reduced AUDPC value of Phytophthora blight compared to the non-treated control. PGPR strains IN937b and SE76 consistently provided significant (*P* ≤ 0.05) protection against *P. capsici* in all three trials (Table 2-4). When plants were treated with IN937b and SE76,
symptoms of crown rot caused by *P. capsici* developed more slowly than it did on non-treated control plants. PGPR strains SE56, 1PC-11, T4 and GB03 significantly reduced the AUDPC value in two of the three trials, PGPR strains SE52, INR7, IN937a, SE49, 1PN-19, and Companion significantly reduced the disease AUDPC in one of the three trials, whereas the strains from commercial products Actinovate AG, BU EXP 1216S, BU EXP 1216C, and SE34 were the least effective treatments for suppressing Phytophthora blight on squash in this greenhouse study (Table 2-4 and Table 2-5). However, no PGPR completely protected the plants from infection by *P. capsici*. At the end of the evaluation, plants were dead in all treatments except for the standard fungicide Presidio®.

**Effect of PGPR on Plant Growth in the Greenhouse**

When fertilization was adequate for plant growth, there was no difference in all treatments concerning growth parameters (shoot and root dry weights, shoot height, root length, etc.) (Table 2-6). Strain IN937a was added as a positive control because it was shown to improve tomato plants growth (Adesemoye et al., 2009a). Mean plant height (33.0 cm), stem caliper growth (6.6 mm), fresh shoot weight (34.8 g) and dry shoot weight (4.2 g) observed in IN937a treated plants were highest in all treatments (Table 2-6). IN937b numerically increased plant height by 4.5% when compared with the non-treated control, albeit not significantly. Dry root weight in IN937b and SE76 increased by 50% compared with the non-treated control. Interestingly, IN937b and SE76 significantly reduced root length by 12% and 7%, respectively. This indicated that IN937b and SE76 did not result in the longest roots, but produced more lateral roots. The ability of plant growth promotion by IN937b appears to be, at least partially, due to the change of root architecture such as lateral roots to increase nutrient and water
uptake by the roots. The ASM treatment did not increase dry root weight or root length indicating that ASM did not improve lateral root production.

When no fertilizer was added, squash treated with IN937b had significantly greater dry shoot and root weight compared to the non-treated control (Figure 2-3). In the experiment to evaluate the effect of fertilization with PGPR treatment on plant growth promotion, no significant difference in plant growth was observed among strains and the non-treated control after fertilizer applied, which are consistent with the result in the Table 2-6.

**Effect of Concentration of Selected PGPR on Disease Suppression**

Application of IN937b at $10^6$, $10^7$ and $10^8$ CFU/mL significantly reduced Phytophthora blight severity compared to the non-treated control. IN937b applied at $10^8$ CFU/mL significantly suppressed disease development by 45% compared to the non-treated control based on the AUDPC values (Table 2-7). When the concentration was reduced to $10^5$ CFU/mL, disease development was similar to that in non-treated control plants. A significant positive correlation ($r^2=0.92$) was found between the IN937b inoculum concentrations and AUDPC value (Figure 2-2).

SE76 at $10^8$ CFU/mL had significantly lower AUDPC values when compared with the untreated control (Table 2-8). Similarly as IN937b, treatment with SE76 at the highest concentration ($10^8$ CFU/mL) had the highest efficacy to reduce disease severity by 42% 6 days after inoculation, which was the last day of the investigation.

IN937a had significant disease control in one of the three trials (Table 2-4). IN937a at high concentrations ($10^8$ and $10^7$ CFU/mL) showed disease symptoms earlier and had greater AUDPC values than the non-treated control (Table 2-9). In this trial, the low concentration ($10^5$ and $10^6$ CFU/mL) significantly reduced disease severity by 25%
and 27%, respectively, compared to the non-treated control. In this study, there is no significant correlation ($r^2=0.022$) between the IN937a inoculum concentrations and AUDPC value (Figure 2-2).

**Effect of Application Timing of PGPR on Disease Development and Age-related Resistance to Phytophthora Crown and Root Rot on Squash Treated with IN937b**

Symptom development of crown and root rot in squash treated with IN937b varied with the timing of application. When IN937b was applied at growth stage 1 (10 days after plant emergence) and stage 2 (17 days after plant emergence), symptoms developed as early as 3 days after inoculation. Water-soaked lesions formed initially on the young seedlings causing the whole plant to wilt quickly (Table 2-10 and 2-11); whereas when applied at the growth stage 3 (24 days after emergence), symptom development was delayed, and symptoms were first visible 7 days after inoculation (Table 2-12). Lesions had visible signs of *P. capsici*, including mycelia and sporangiophores with sporangia being easily found in the late stage of development.

IN937b and SE76 significantly reduced disease severity compared to the untreated control when they were applied at all growth stages. In the trial with best control efficacy, IN937b and SE76 significantly suppressed the disease by 54% and 57%, respectively, when they were applied at the stage 2 i.e. the first PGPR application was made at 17 days after seeding (Table 2-11).

**Effect of PGPR on Chlorophyll Content in Squash Leaves**

The chlorophyll content (as estimated with a SPAD meter) is an indirect indicator of plant health. The chlorophyll content was significantly greater in leaves of IN937b and ASM (Actigard) treated plants when compared to the untreated (Figure 2-4). There was
no difference in chlorophyll content among the treatments of SE76, IN937a and the non-treated control.

Five days after inoculation, significant differences in chlorophyll content were observed in *P. capsici* infected plants in the treatments SE76 and ASM compared with the untreated control (Table 2-13). Damage of roots and stems led to a drastic decrease in SPAD values in the leaves of IN937a treated plants and the non-treated control. On the other hand, plant treated with IN937b showed only a moderate decrease compared with SE76 and ASM treatments, although IN937b treated plant did not show any symptoms at the time of data collection.

**Discussion**

In the present study, select *Bacillus* PGPR strains (IN937b, SE76, SE56, SE49, 1PC-11, and GB03) showed effective biological control activity against *P. capsici* which causes crown and root rot on squash in this greenhouse. We also investigated the effect of *Bacillus* PGPR strains IN937b, SE76 and IN937a on squash growth promotion. However, the growth promotion effect only occurred under conditions without fertilization. Moreover, in our system, PGPR strains IN937b and SE76 were consistent in improving the performance in managing of Phytophthora crown and root rot on squash, when applied at the concentration of $10^8$ CFU/mL and a longer time period i.e. the first PGPR application was made at 17 days after seeding.

**Virulence Differentiation of *P. capsici* Isolates**

In this study, the susceptibility of the squash cultivar, Senator, to Phytophthora crown and root rot differed significantly among three isolates 121, 146, and 151. Based on the results of disease severity and AUDPC value, there appeared to be differences in virulence among these *P. capsici* isolates. Isolate 121 was highly virulent, causing
crown and root rot. Isolate 146 was the least virulent based on slow symptom
development and lower disease incidence compared to plants treated with the other two
isolates. Different levels of virulence among *P. capsici* isolates led to similar conclusions
in previous studies with pepper (Oelke et al., 2003). Knowing that different levels of
virulence exist among *P. capsici* isolates infecting squash, we used isolate 151 with
moderate virulence caused symptoms to develop and tissue to collapse more than one
week after inoculation in the greenhouse.

In our tests, zoospores of *P. capsici* were used for inoculation. The development
of Phytophthora crown and root rot was affected by inoculum density (CafeFilho and
Duniway, 1996). When *P. capsici* was tested at 1, 2, 4, 6, 8, and 10 × 10^4 zoospores/mL
to determine the optimum concentration for the future experiments, high levels of crown
and root rot developed, and plant disease increased very quickly the first week after
inoculation when the concentrations were 6, 8, and 10 × 10^4 zoospores/mL. However,
only low levels of crown and root rot developed, and disease did not develop in some
plants inoculated with 1 × 10^4 zoospores/mL. Optimum disease levels were observed in
plants inoculated with *P. capsici* at the concentrations of 2 to 8 × 10^4 zoospores/mL.
Consequently, 2 × 10^4 zoospores/mL was chosen for further greenhouse experiments to
guaranty optimum plant infection and symptom development.

**Control of Phytophthora Crown and Root Rot by Bacillus spp.**

The development of biological control agents (BCAs) effective for control of
Phytophthora blight depends on the selection of an efficient strain, the introduction of
adequate bacterial cells into the soil, the interaction between the pathogen and the bio-
control strain, and the timing and method of application. In general, introducing large
populations of BCAs might provide a competitive advantage over residential
microorganisms. Sometimes, the bacterial strains that showed the greatest potential for plant growth promotion were not necessarily the best for inhibiting pathogens. *Bacillus* plays an important role in the control of soil-borne plant pathogens such as in suppressing of crown and root rot caused by *P. capsici* (Zhang et al., 2010). Competition for nutrients and space between PGPR and the pathogen is considered to be one of the mechanisms whereby PGPR biologically control plant diseases.

In our greenhouse trials, some PGPR strains provided strong suppression of Phytophthora crown and root rot, whereas others showed only moderate activity or no activity at all. Two PGPR strains IN937b and SE76 consistently showed significant suppression of Phytophthora blight on squash, which agrees with earlier studies (Zhang et al., 2010). These *Bacillus* strains were most effective among the PGPR strains tested based on data from our greenhouse trials. PGPR strains IN937b and SE76 showed strong disease suppression, which may make them promising candidates for the development of BCAs that manage Phytophthora blight on squash. In particular, *B. subtilis* IN937b has shown potential to suppress disease and to promote plant growth, which makes it a potentially promising BCAs for management of Phytophthora blight on squash.

The effect of disease suppression by IN937b and SE76 was greater at high concentrations (10⁸ CFU/mL) than low concentrations (10⁷ and 10⁶ CFU/mL), although these two PGPR strains applied at low concentrations were also effective in some of the greenhouse trials. The efficacy of the lowest concentration (10⁵ CFU/mL) was decreased due to the lack of adequate viable cells for optimal control of crown rot.
Similar results were reported when $10^5$ CFU/g root was present in the case of \textit{Pseudomonas} spp. (Raaijmakers et al., 1995a).

\textbf{Enhanced Plant Growth by \textit{Bacillus} spp.}

The definition of PGPR comes from the fact that PGPR are able to increase plant growth. Improvements of plant growth by PGPR on many different crops are well documented (Kloepper et al., 2004). The ability of \textit{Bacillus} strains IN937b, SE76 and IN937a to increase squash plant biomass only occurred in the present study only when plants were not fertilized, indicating that plant growth was strongly influenced by fertilization. When the nutrient concentrations in the soil were sufficient, it was difficult to promote plant growth by application of \textit{Bacillus} PGPR. On the other hand, \textit{Bacillus} spp. could reduce fertilizer application rates due to its ability to increase nutrients available to crops without any significant reduction of yield (Adesemoye et al., 2009b).

IN937b had a positive effect on plant growth, especially for lateral root growth. Root biomass is commonly used to indicate growth promotion by PGPR and root functions such as water and nutrient uptake (Lopez-Bucio et al., 2003). The increase in lateral root formation by PGPR strains helps to explain the observed growth promotions. Nutrient uptake by plant generally depends on the amount of root surface area (Simunek and Hopmans, 2009).

\textbf{Relation of Leaf Chlorophyll Content and Plant Health}

The present study showed that chlorophyll content increased in leaves of IN937b treated plants under greenhouse conditions, which agrees with the previous report indicating that treating lettuce leaves with PGPR increased leaf chlorophyll content (Arkhipova et al., 2005). In this present study, the increase in chlorophyll content in IN937b treated squash plants indicated that foliar nitrogen content in plant leaves was
increased by IN937b, which might indirectly influence plant growth improvement. The increase in chlorophyll content might be due to the changes in the plant’s metabolism.

Five days after inoculation with *P. capsici*, squash plants began to show symptoms of water-soaked lesions on the stem, especially the leaves turned yellow. Treatments with PGPR strain SE76 significantly reduced disease severity compared with the untreated control, while leaf chlorophyll content was also significantly higher than in the untreated control. IN937a had no effect on disease suppression and similar leaf chlorophyll content as the untreated control.

**Optimization of Application Rate and Timing**

Loss of biocontrol efficiency observed after 2 weeks is associated with a decrease in the populations of PGPR (Strigul and Kravchenko, 2006). Maintain a high number of PGPR as well as the timing of applications is possible to obtain an optimal long-term control of diseases. This requires to assess the behavior of a specific BCA. Understanding the behavior of a BCA in the environment may lead to improved performance of the BCA, including nutrient use (Ji et al., 1997) and site preference (Fan et al., 2011). If certain behaviors can be correlated with better disease control, then it may be possible to promote these behaviors and, hence, improve disease control. BCAs can be made into more reliable and predictable products by promoting the behaviors associated with improved disease control and by optimizing application methods.

Our results indicated that $10^8$ CFU/mL is the optimum concentration for PGPR strains IN937b and SE76 to consistently promote plant growth and suppress disease development. Bacterial concentrations of PGPR above $10^8$ CFU/mL were not tested
since the highest vegetative cells of PGPR strains cultured within 24 h in NB are approximately $1 \times 10^8$ CFU/mL.

It is important to determine a threshold of inoculum density for IN937b and SE76 at which they significantly suppress the Phytophthora blight on squash. Compared to the low concentration of $10^5$ CFU/mL, treatments with PGPR at high concentrations demonstrated greater control efficacy. This is because symptom development was delayed due to the high population of Bacillus spp. in the potting mix which may play a long-term role in disease suppression. Some studies have suggested that specific PGPR strains secreted secondary metabolites to prevent pathogen invasion and spread (Compant et al., 2013). Thus, the low severity of disease in PGPR treatments was probably due to low frequency of infection by the pathogen, reducing the potential for pathogens to attack and colonize the root tissues of plants (Nihorimbere et al., 2010). In general, two applications of PGPR were generally more effective than one treatment, since the seedling stage highlighted the importance of maintaining a high population of PGPR in the soil. This high population may reduce infection by pathogens (Poleatewich et al., 2012). This requirement, however, poses a challenge to maintain high PGPR populations in the soil because vegetative cells of PGPR strains lose efficacy quickly under dry soil conditions due to poor colonization (West et al., 1985). Repeated applications of PGPR may enhance the survival of PGPR in the soil (Schmidt et al., 2004). In the present study, treatments of two applications likely helped to maintain high populations of PGPR throughout the experiments.

The effect of age-related resistance in squash plants treated with PGPR remains unknown (Sunwoo et al., 1996). Results from our study demonstrated that the timing of
applying PGPR can be crucial in the efficacy of PGPR against infection by *P. capsici*. Application to the stage 2 (stage 1 was the youngest stage) seedlings was remarkably more efficient in disease suppression than when it was applied at the two older stages, suggesting that the PGPR strains IN937b and SE76 exhibited strong reactions starting from early growth stage. It also suggested that PGPR strains need time to establish a relationship with the host plant. In particular, the younger seedlings developed more crown rot disease, similar to that described by Morin for gorse (*Ulex europaeus* L.) plants (Morin et al., 1998). The gorse plants tested were susceptible to pathogens, but younger plants were more easily killed. The age of plants influenced the capability of pathogens to infect and spread due to thickening of the cuticle and inducing resistance response. However, the effectiveness of *Bacillus* strains in suppressing pathogens decreases as plants mature.

During the past decade, several studies have focused on bio-control activities of *B. subtilis* in controlling plant diseases. Antimicrobial metabolites are often associated with BCAs in the genus *Bacillus*, which may act directly on the pathogen. Indigenous bacteria that are antagonistic to plant pathogens in the soil could make a substantial contribution to preventing plant diseases, and therefore represent an alternative to using chemical pesticides. It is clear that BCAs are still too variable in their performance to be successfully used as a common practice for disease management. This inconsistency has been attributed to a number of factors including fluctuations in the environment and the variable expression of genes involved in disease suppression and root colonization by the BCA. The present suggests that further investigations are needed regarding the role of environmental factors in modulating performance of *Bacillus* PGPR strains in
order to improve their efficacy and performance in suppressing phytopathogens under field conditions. A better understanding of the bio-control mechanisms may be helpful for guiding future efforts in BCA development.
Table 2-1. Relevant information for PGPR strains tested in this study

<table>
<thead>
<tr>
<th>Tested PGPR strains a</th>
<th>Identity</th>
<th>Protected plant</th>
<th>Target pathogen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE34</td>
<td><em>B. safensis</em></td>
<td>Rice</td>
<td><em>Xanthomonas oryzae</em> pv. oryzae</td>
<td>(Chithrashree et al., 2011)</td>
</tr>
<tr>
<td>SE49</td>
<td><em>B. safensis</em></td>
<td>Cucumber</td>
<td><em>Colletotrichum orbiculare</em></td>
<td>(Wei et al., 1996)</td>
</tr>
<tr>
<td>SE52</td>
<td><em>B. safensis</em></td>
<td>Loblolly pine</td>
<td><em>Cronartium quercuum</em> f. sp. fusiforme</td>
<td>(Enebak and Carey, 2000)</td>
</tr>
<tr>
<td>SE56</td>
<td><em>Lysinibacillus boronitolerans</em></td>
<td>Pepper</td>
<td><em>Colletotrichum gloeosporioides</em></td>
<td>(Jetiyanon and Kloepper, 2002)</td>
</tr>
<tr>
<td>SE76</td>
<td><em>B. safensis</em></td>
<td>Tomato</td>
<td><em>Colletotrichum</em> gloeosporioides Ralstonia solanearum</td>
<td>(Jetiyanon, 2007)</td>
</tr>
<tr>
<td>IN937a</td>
<td><em>B. subtilis</em></td>
<td>Tomato</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IN937b</td>
<td><em>B. subtilis</em></td>
<td>Pepper</td>
<td>Sclerotium rolfsii</td>
<td>(Jetiyanon, 2007)</td>
</tr>
<tr>
<td>INR7</td>
<td><em>B. pumilus</em></td>
<td>Pepper</td>
<td><em>Xanthomonas axonopodis</em> pv. vesicatoria</td>
<td>(Yi et al., 2013)</td>
</tr>
<tr>
<td>T4</td>
<td><em>B. safensis</em></td>
<td>Tobacco</td>
<td>Peronospora tabacina</td>
<td>(Zhang et al., 2002)</td>
</tr>
<tr>
<td>GB03</td>
<td><em>B. subtilis</em></td>
<td>Arabidopsis thaliana</td>
<td>Pseudomonas syringae pv. tomato DC3000</td>
<td>(Kumar et al., 2012)</td>
</tr>
<tr>
<td>1PC-11</td>
<td><em>B. macauensis</em></td>
<td>Squash</td>
<td>Phytophthora capsici</td>
<td>(Zhang et al., 2010)</td>
</tr>
<tr>
<td>1PN-19</td>
<td><em>B. subtilis</em></td>
<td>Squash</td>
<td>Phytophthora capsici</td>
<td>(Zhang et al., 2010)</td>
</tr>
<tr>
<td>Companion (GB03)</td>
<td><em>B. subtilis</em></td>
<td>Arabidopsis thaliana</td>
<td>Pseudomonas syringae pv. tomato DC3000</td>
<td>(Kumar et al., 2012)</td>
</tr>
<tr>
<td>BU EXP 1216S (MBI600)</td>
<td><em>B. subtilis</em></td>
<td>Rice</td>
<td>Rhizoctonia solani</td>
<td>(Kumar et al., 2010)</td>
</tr>
<tr>
<td>BU EXP 1216C (MBI600)</td>
<td><em>B. subtilis</em></td>
<td>Squash</td>
<td>Podosphaera xanthii</td>
<td>(Zhang et al., 2011)</td>
</tr>
<tr>
<td>Actinovate AG</td>
<td><em>Streptomyces lydicus WYEC 108</em></td>
<td>Soybean</td>
<td>Sclerotinia sclerotiorum</td>
<td>(Zeng et al., 2012a)</td>
</tr>
</tbody>
</table>

a PGPR strains were provided by Dr. Joseph W. Kloepper.
Table 2-2. Disease development in squash inoculated with different isolates of *P. capsici*

<table>
<thead>
<tr>
<th>Zoospore concentration $^a$</th>
<th>Disease severity $^b$</th>
<th>AUDPC $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>121</td>
<td>146</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0.0  b $^d$</td>
<td>0.0  b</td>
</tr>
<tr>
<td>$10^3$</td>
<td>3.3  a</td>
<td>0.0  b</td>
</tr>
<tr>
<td>$10^4$</td>
<td>5.0  a</td>
<td>0.0  b</td>
</tr>
<tr>
<td>$10^5$</td>
<td>5.0  a</td>
<td>3.8  a</td>
</tr>
</tbody>
</table>

$^a$ Zoospore solutions of three isolates (121, 146, 151) were applied in the pots as a soil drench when plants had four completely expanded leaves. Each plant was inoculated with 5 mL of *P. capsici* inoculum.

$^b$ Disease severity was rated based on the scale from 0 (healthy) to 5 (dead). Data were collected 7 days after pathogen inoculation.

$^c$ AUDPC was calculated based on the disease severity rated 5, 6, and 7 days after pathogen inoculation

$^d$ In each column in disease severity, each value represents the mean of four replicates. Values followed by the same letter are not significantly different according to Fisher’s protected Least Significant Difference (LSD) test ($P = 0.05$).
Table 2-3. Effect of the concentration of *P. capsici* isolate 151 on disease development

<table>
<thead>
<tr>
<th>Zoospore concentration (×10^4) a</th>
<th>Disease severity b</th>
<th>Time after inoculation (Day)</th>
<th>AUDPC c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6 a d</td>
<td>3.0 a</td>
<td>3.0 b</td>
</tr>
<tr>
<td>2</td>
<td>0.6 a</td>
<td>3.0 a</td>
<td>3.8 ab</td>
</tr>
<tr>
<td>4</td>
<td>0.8 a</td>
<td>3.0 a</td>
<td>3.5 ab</td>
</tr>
<tr>
<td>6</td>
<td>1.1 a</td>
<td>2.8 a</td>
<td>3.3 b</td>
</tr>
<tr>
<td>8</td>
<td>1.8 a</td>
<td>3.0 a</td>
<td>4.5 a</td>
</tr>
<tr>
<td>10</td>
<td>1.8 a</td>
<td>4.0 a</td>
<td>4.5 a</td>
</tr>
</tbody>
</table>

a The concentration of *P. capsici* inoculum was diluted to 1, 2, 4, 6, 8, and 10 × 10^4 zoospores/mL. Squash plants were inoculated with 5 mL of *P. capsici* inoculum.

b Disease severity was rated based on the scale from 0 (healthy) to 5 (dead).

c AUDPC was calculated based on the disease severity rated 5, 7, and 9 days after pathogen inoculation.

d Each value represents the mean of four replicates. Means followed by the same letter within a column are not significantly different according to Fisher's protected LSD test (*p* = 0.05).
Table 2-4. Efficacy of PGPR (1-8 PGPR strains) on suppression of Phytophthora crown and root rot under greenhouse conditions

<table>
<thead>
<tr>
<th>Treatment a</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE34</td>
<td>14.4 a c</td>
<td>17.4 ab</td>
<td>24.8 a</td>
</tr>
<tr>
<td>SE52</td>
<td>10.0 b</td>
<td>15.6 b</td>
<td>24.4 a</td>
</tr>
<tr>
<td>SE56</td>
<td>12.0 ab</td>
<td>14.7 b</td>
<td>20.1 b</td>
</tr>
<tr>
<td>SE76</td>
<td>5.8 c</td>
<td>15.6 b</td>
<td>17.1 c</td>
</tr>
<tr>
<td>INR7</td>
<td>13.6 a</td>
<td>15.3 b</td>
<td>22.9 a</td>
</tr>
<tr>
<td>1PN-19</td>
<td>13.6 a</td>
<td>15.3 b</td>
<td>23.7 a</td>
</tr>
<tr>
<td>IN937a</td>
<td>13.6 a</td>
<td>15.9 b</td>
<td>23.9 a</td>
</tr>
<tr>
<td>IN937b</td>
<td>5.6 c</td>
<td>15.3 b</td>
<td>19.6 b</td>
</tr>
<tr>
<td>Presidio®</td>
<td>1.0 d</td>
<td>0.0 c</td>
<td>0.0 d</td>
</tr>
<tr>
<td>Non-treated control</td>
<td>12.8 ab</td>
<td>19.2 a</td>
<td>23.9 a</td>
</tr>
</tbody>
</table>

a PGPR strains were applied twice (1.0 \times 10^8 CFU/mL; 20mL) as a soil drench. Presidio® (1.2 mL/L; 20mL) was applied as a standard fungicide which provide control of Phytophthora blight.

b *P. capsici* inoculum on crown and roots was diluted to 2 \times 10^4 zoospores/mL. Plants were inoculated by 5 mL of *P. capsici* inoculum. Disease severity was collected based on the rating scale from 0 (healthy) to 5 (dead). AUDPC calculated based on the disease severity. Trial 1: 11, 13, and 15 days after pathogen inoculation; Trial 2: 9, 12, and 15 days after pathogen inoculation; Trial 3: 7, 9, and 14 days after pathogen inoculation.

c Each value represents the mean of five replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Table 2-5. Efficacy of PGPR (9-16 PGPR strains) on suppression of Phytophthora crown and root rot under greenhouse conditions

<table>
<thead>
<tr>
<th>Treatment a</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE49</td>
<td>15.6 ab c</td>
<td>10.3 bc</td>
<td>5.8 b</td>
</tr>
<tr>
<td>Com</td>
<td>9.6 cd</td>
<td>12.4 abcd</td>
<td>8.6 ab</td>
</tr>
<tr>
<td>AG</td>
<td>14.4 ab</td>
<td>16.1 a</td>
<td>8.4 ab</td>
</tr>
<tr>
<td>GB03</td>
<td>8.6 d</td>
<td>7.8 c</td>
<td>8.8 ab</td>
</tr>
<tr>
<td>1216S</td>
<td>14.4 ab</td>
<td>13.2 abc</td>
<td>8.4 ab</td>
</tr>
<tr>
<td>1216C</td>
<td>14.8 ab</td>
<td>13.8 ab</td>
<td>9.6 ab</td>
</tr>
<tr>
<td>T4</td>
<td>12.6 bcd</td>
<td>9.8 bc</td>
<td>11.4 a</td>
</tr>
<tr>
<td>1PC-11</td>
<td>13.6 bcd</td>
<td>11.7 bcd</td>
<td>8.2 ab</td>
</tr>
<tr>
<td>Presidio®</td>
<td>0.0 e</td>
<td>0.0 d</td>
<td>0.0 c</td>
</tr>
<tr>
<td>Non-treated control</td>
<td>18.2 a</td>
<td>16.8 a</td>
<td>10.2 ab</td>
</tr>
</tbody>
</table>

a PGPR strains were applied twice (1.0 ×10⁸ CFU/mL; 20mL) as a soil drench. Presidio® (1.2 mL/L; 20mL) was applied as a standard fungicide which provide control of Phytophthora blight.

b P. capsici inoculum on crown and roots was diluted to 2 × 10⁴ zoospores/mL. Plants were inoculated by 5 mL of P. capsici inoculum. Disease severity was collected based on the rating scale from 0 (healthy) to 5 (dead). AUDPC calculated based on the disease severity. Trial 1: 11, 15, and 17 days after pathogen inoculation; Trial 2: 12, 14, and 17 days after pathogen inoculation; Trial 3: 7, 9, and 11 days after pathogen inoculation.

c Each value represents the mean of five replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Table 2-6. Effect of PGPR on plant growth under greenhouse conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant height cm</th>
<th>Stem diameter mm</th>
<th>Fresh shoot g</th>
<th>Dry shoot g</th>
<th>Fresh root g</th>
<th>Dry root g</th>
<th>Root elongation cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN937b</td>
<td>32.3 ab</td>
<td>6.4 a</td>
<td>33.6 ab</td>
<td>3.9 a</td>
<td>4.6 a</td>
<td>0.3 a</td>
<td>14.3 c</td>
</tr>
<tr>
<td>SE76</td>
<td>31.6 ab</td>
<td>6.2 a</td>
<td>34.0 ab</td>
<td>3.9 a</td>
<td>4.3 a</td>
<td>0.3 a</td>
<td>15.0 bc</td>
</tr>
<tr>
<td>IN937a</td>
<td>33.0 a</td>
<td>6.6 a</td>
<td>34.8 a</td>
<td>4.2 a</td>
<td>4.3 a</td>
<td>0.2 a</td>
<td>15.3 b</td>
</tr>
<tr>
<td>Actigard®</td>
<td>32.9 a</td>
<td>6.5 a</td>
<td>32.6 ab</td>
<td>4.1 a</td>
<td>4.3 a</td>
<td>0.2 a</td>
<td>14.4 c</td>
</tr>
<tr>
<td>Non-treated control</td>
<td>30.9 ab</td>
<td>6.3 a</td>
<td>34.1 ab</td>
<td>4.0 a</td>
<td>4.4 a</td>
<td>0.2 a</td>
<td>16.2 a</td>
</tr>
<tr>
<td>Non-treated control (-F)</td>
<td>30.3 b</td>
<td>6.2 a</td>
<td>30.7 b</td>
<td>2.9 b</td>
<td>3.3 b</td>
<td>0.1 b</td>
<td>13.3 d</td>
</tr>
</tbody>
</table>

a PGPR strains were applied twice (1.0 × 10^8 CFU/mL; 20 mL) as a soil drench. Actigard® (30 mg/L; 20 mL) was applied as a positive SAR inducer. Water soluble fertilizer 20:20:20 once a week (3.5 g/L; 20 mL per plant) was applied to all treatments except for the untreated control (-F). The untreated control (-F) represents the plants grown without fertilization.

b Plants were investigated at 40 days after planting, the root and shoot were separated, and the biomass was measured. The length of root tissues was recorded to determine the root elongation.

c Each value represents the mean of ten replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Table 2-7. Effect of IN937b concentration on crown and root rot disease

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease severity b</th>
<th>AUDPC c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time after inoculation (Day)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>$10^5$ CFU/mL</td>
<td>0.3 a</td>
<td>1.4 b</td>
</tr>
<tr>
<td>$10^6$ CFU/mL</td>
<td>0.1 b</td>
<td>1.2 b</td>
</tr>
<tr>
<td>$10^7$ CFU/mL</td>
<td>0.0 b</td>
<td>1.0 b</td>
</tr>
<tr>
<td>$10^8$ CFU/mL</td>
<td>0.0 b</td>
<td>1.0 b</td>
</tr>
<tr>
<td>Non-treated control</td>
<td>0.0 b</td>
<td>2.1 a</td>
</tr>
<tr>
<td>Actigard®</td>
<td>0.0 b</td>
<td>0.0 c</td>
</tr>
<tr>
<td>Presidio®</td>
<td>0.0 b</td>
<td>0.0 c</td>
</tr>
</tbody>
</table>

a PGPR were applied twice as a soil drench. The concentration of IN937b was adjusted to $10^5$, $10^6$, $10^7$, and $10^8$ CFU/mL, and 20 mL was applied to each plant. Presidio® (1.2 mL/L; 20mL/plant) and Actigard® (30 mg/L; 20mL/plant) were applied as a positive fungicide and SAR control, respectively, of Phytophthora blight.

b P. capsici inoculum on crown and roots was diluted to $2 \times 10^4$ zoospores/mL. Plants were inoculated by 5 mL of P. capsici inoculum. Disease severity was collected at 3, 4, 5, and 6 days after pathogen inoculation based on the rating scale from 0 (healthy) to 5 (dead).

c AUDPC calculated based on the disease severity 3, 4, 5, and 6 days after pathogen inoculation.

d Each value represents the mean of ten replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05).
### Table 2-8. Effect of SE76 concentration on crown and root rot disease

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease severity b</th>
<th>AUDPC c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time after inoculation (Day)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>$10^5$ CFU/mL</td>
<td>0.0 b d</td>
<td>1.0 a</td>
</tr>
<tr>
<td>$10^6$ CFU/mL</td>
<td>0.5 a</td>
<td>1.1 a</td>
</tr>
<tr>
<td>$10^7$ CFU/mL</td>
<td>0.0 b</td>
<td>1.5 a</td>
</tr>
<tr>
<td>$10^8$ CFU/mL</td>
<td>0.0 b</td>
<td>1.1 a</td>
</tr>
<tr>
<td>Non-treated control</td>
<td>0.0 b</td>
<td>1.8 a</td>
</tr>
<tr>
<td>Actigard®</td>
<td>0.0 b</td>
<td>0.0 b</td>
</tr>
<tr>
<td>Presidio®</td>
<td>0.0 b</td>
<td>0.0 b</td>
</tr>
</tbody>
</table>

**a** PGPR were applied twice as a soil drench. The concentration of SE76 was adjusted to $10^5$, $10^6$, $10^7$, and $10^8$ CFU/mL, and 20 mL was applied to each plant. Presidio® (1.2 mL/L; 20mL/plant) and Actigard® (30 mg/L; 20mL/plant) were applied as a positive fungicide and SAR control, respectively, of Phytophthora blight.

**b** *P. capsici* inoculum on crown and roots was diluted to $2 \times 10^4$ zoospores/mL. Plants were inoculated by 5 mL of *P. capsici* inoculum. Disease severity was collected at 3, 4, 5, and 6 days after pathogen inoculation based on the rating scale from 0 (healthy) to 5 (dead).

**c** AUDPC calculated based on the disease severity 3, 4, 5, and 6 days after pathogen inoculation.

**d** Each value represents the mean of ten replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05).
<table>
<thead>
<tr>
<th>Treatment a</th>
<th>Disease severity b</th>
<th>Time after inoculation (Day)</th>
<th>AUDPC c</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁵ CFU/mL</td>
<td>0.0 b d</td>
<td>1.3 c</td>
<td>1.6 b</td>
</tr>
<tr>
<td>10⁶ CFU/mL</td>
<td>0.0 b</td>
<td>1.6 bc</td>
<td>1.6 b</td>
</tr>
<tr>
<td>10⁷ CFU/mL</td>
<td>0.4 a</td>
<td>2.2 a</td>
<td>2.8 a</td>
</tr>
<tr>
<td>10⁸ CFU/mL</td>
<td>0.5 a</td>
<td>2.3 a</td>
<td>2.6 a</td>
</tr>
<tr>
<td>Non-treated control</td>
<td>0.0 b</td>
<td>1.9 ab</td>
<td>2.4 a</td>
</tr>
<tr>
<td>Actigard®</td>
<td>0.0 b</td>
<td>0.0 d</td>
<td>0.0 c</td>
</tr>
<tr>
<td>Presidio®</td>
<td>0.0 b</td>
<td>0.0 d</td>
<td>0.0 c</td>
</tr>
</tbody>
</table>

a PGPR were applied twice as a soil drench. The concentration of IN937a was adjusted to 10⁵, 10⁶, 10⁷, and 10⁸ CFU/mL, and 20 mL was applied to each plant. Presidio® (1.2 mL/L; 20mL/plant) and Actigard® (30 mg/L; 20mL/plant) were applied as a positive fungicide and SAR control, respectively, of Phytophthora blight.

b P. capsici inoculum on crown and roots was diluted to 2 × 10⁴ zoospores/mL. Plants were inoculated by 5 mL of P. capsici inoculum. Disease severity was collected at 3, 4, 5, and 6 days after pathogen inoculation based on the rating scale from 0 (healthy) to 5 (dead).

c AUDPC calculated based on the disease severity 3, 4, 5, and 6 days after pathogen inoculation.

d Each value represents the mean of ten replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Table 2-10. Effect of *Bacillus* spp. application timing (stage 1: seed) on crown and root rot disease

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease severity</th>
<th>( \text{Time after inoculation (Day)} )</th>
<th>AUDPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( 3 )</td>
<td>( 5 )</td>
<td>( 6 )</td>
</tr>
<tr>
<td>IN937b</td>
<td>0.4 b</td>
<td>1.4 b</td>
<td>2.8 bc</td>
</tr>
<tr>
<td>SE76</td>
<td>0.0 b</td>
<td>1.1 b</td>
<td>2.5 c</td>
</tr>
<tr>
<td>IN937a</td>
<td>0.5 b</td>
<td>2.5 a</td>
<td>3.6 ab</td>
</tr>
<tr>
<td>Non-treated control</td>
<td>1.3 a</td>
<td>3.0 a</td>
<td>3.9 a</td>
</tr>
<tr>
<td>Actigard(^\circ)</td>
<td>0.0 b</td>
<td>0.0 c</td>
<td>0.0 d</td>
</tr>
<tr>
<td>Presidio(^\circ)</td>
<td>0.0 b</td>
<td>0.0 c</td>
<td>0.0 d</td>
</tr>
</tbody>
</table>

\(^a\) PGPR strains were applied twice (seed soaking and 10 days after emergence) as a soil drench (1.0 \( \times \) \( 10^8 \) CFU/mL; 20mL). Presidio\(^\circ\) (1.2 mL/L; 20mL) and Actigard\(^\circ\) (30 mg/L; 20mL/plant) was applied as the positive control of Phytophthora blight.

\(^b\) *P. capsici* inoculum on crown and roots was diluted to \( 2 \times 10^4 \) zoospores/mL. Plants were inoculated by 5 mL of *P. capsici* inoculum. Disease severity was collected at 3, 5, 6, and 7 days after pathogen inoculation based on the rating scale from 0 (healthy) to 5 (dead).

\(^c\) AUDPC calculated based on the disease severity 3, 5, 6, and 7 days after pathogen inoculation.

\(^d\) Each value represents the mean of ten replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test \( (p = 0.05) \).
Table 2-11. Effect of *Bacillus* spp. application timing (stage 2: 10 days after emergence) on crown and root rot disease

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease severity b</th>
<th>Time after inoculation (Day)</th>
<th>AUDPC c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>IN937b</td>
<td>0.8 ab d</td>
<td>1.2 b</td>
<td>1.2 b</td>
</tr>
<tr>
<td>SE76</td>
<td>0.7 bc</td>
<td>1.2 b</td>
<td>1.3 b</td>
</tr>
<tr>
<td>IN937a</td>
<td>1.6 a</td>
<td>2.5 a</td>
<td>2.7 a</td>
</tr>
<tr>
<td>Non-treated control</td>
<td>1.2 ab</td>
<td>3.0 a</td>
<td>3.5 a</td>
</tr>
<tr>
<td>Actigard®</td>
<td>0.0 c</td>
<td>0.0 c</td>
<td>0.0 c</td>
</tr>
<tr>
<td>Presidio®</td>
<td>0.0 c</td>
<td>0.0 c</td>
<td>0.0 c</td>
</tr>
</tbody>
</table>

a PGPR strains were applied twice (10 days and 17 days after emergence) as a soil drench (1.0 × 10^8 CFU/mL; 20mL). Presidio® (1.2 mL/L; 20mL) and Actigard® (30 mg/L; 20mL/plant) was applied as the positive control of Phytophthora blight.

b *P. capsici* inoculum on crown and roots was diluted to 2 × 10^4 zoospores/mL. Plants were inoculated by 5 mL of *P. capsici* inoculum. Disease severity was collected at 3, 5, 7, and 9 days after pathogen inoculation based on the rating scale from 0 (healthy) to 5 (dead).

c AUDPC calculated based on the disease severity 3, 5, 7, and 9 days after pathogen inoculation.

d Each value represents the mean of ten replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05).
### Table 2-12. Effect of *Bacillus* spp. application timing (Stage 3: 17 days after emergence) on crown and root rot disease

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease severity b</th>
<th>AUDPC c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time after inoculation (Day)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>IN937b</td>
<td>1.4 b</td>
<td>1.4 c</td>
</tr>
<tr>
<td>SE76</td>
<td>1.8 ab</td>
<td>2.1 b</td>
</tr>
<tr>
<td>IN937a</td>
<td>2.0 a</td>
<td>2.1 b</td>
</tr>
<tr>
<td>Non-treated control</td>
<td>1.9 a</td>
<td>2.8 a</td>
</tr>
<tr>
<td>Actigard®</td>
<td>0.5 c</td>
<td>0.7 d</td>
</tr>
<tr>
<td>Presidio®</td>
<td>0.0 d</td>
<td>0.0 e</td>
</tr>
</tbody>
</table>

a PGPR strains were applied twice (17 days and 24 days after emergence) as a soil drench (1.0 × 10^8 CFU/mL; 20mL). Presidio® (1.2 mL/L; 20mL) and Actigard® (30 mg/L; 20mL/plant) was applied as the positive control of Phytophthora blight.
b *P. capsici* inoculum on crown and roots was diluted to 2 × 10^4 zoospores/mL. Plants were inoculated by 5 mL of *P. capsici* inoculum. Disease severity was collected at 7, 9, 11, and 13 days after pathogen inoculation based on the rating scale from 0 (healthy) to 5 (dead).
c AUDPC calculated based on the disease severity 7, 9, 11, and 13 days after pathogen inoculation.
d Each value represents the mean of ten replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Table 2-13. Effect of PGPR on chlorophyll content in squash leaves under greenhouse conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease severity $^b$</th>
<th>SPAD reading $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN937b</td>
<td>0.0 b $^d$</td>
<td>21.7 b</td>
</tr>
<tr>
<td>SE76</td>
<td>0.3 b</td>
<td>29.3 a</td>
</tr>
<tr>
<td>IN937a</td>
<td>1.0 a</td>
<td>16.6 b</td>
</tr>
<tr>
<td>Actigard®</td>
<td>0.0 b</td>
<td>32.9 a</td>
</tr>
<tr>
<td>Non-treated Control</td>
<td>1.0 a</td>
<td>16.8 b</td>
</tr>
</tbody>
</table>

$^a$ PGPR were applied twice as a soil drench. The concentration of IN937b was adjusted to $10^8$ CFU/mL, and 20 mL was applied to each plant. Actigard® (30 mg/L; 20mL/plant) was applied as a positive SAR control of Phytophthora blight.

$^b$ Phytophthora capsici inoculum was diluted to $2 \times 10^4$ zoospores/mL. Plants were inoculated with 5 mL of $P$. capsici inoculum. Disease severity was rated at 5 days after pathogen inoculation based on the rating scale from 0 (healthy) to 5 (dead).

$^c$ The SPAD measurement were carried out 5 days after inoculation. SPAD values were measured on three leaves at the same position on each plant.

$^d$ Each value represents the mean of 5 replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test ($p = 0.05$).
Figure 2-1. Regression analysis of the concentration of *P. capsici* isolate 151 on disease development. AUDPC was calculated based on the disease severity rated 5, 7, and 9 days after pathogen inoculation. Each value represents the mean of four replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test ($p = 0.05$).
Figure 2-2. Regression analysis of Effect of PGPR strains concentration on crown and root rot disease. A: IN937b, B: SE76, C: IN937a. PGPR were applied twice as a soil drench. The concentration of PGPR was adjusted to $10^5$, $10^6$, $10^7$, and $10^8$ CFU/mL, and 20 mL was applied to each plant. Presidio® (1.2 mL/L; 20mL/plant) and Actigard® (30 mg/L; 20mL/plant) were applied as a positive fungicide and SAR control, respectively, of Phytophthora blight. *P. capsici* inoculum on crown and roots was diluted to $2 \times 10^4$ zoospores/mL. Plants were inoculated by 5 mL of *P. capsici* inoculum. Disease severity was collected at 3, 4, 5, and 6 days after pathogen inoculation based on the rating scale from 0 (healthy) to 5 (dead). AUDPC calculated based on the disease severity 3, 4, 5, and 6 days after pathogen inoculation. Values with same letter within a column are not significantly different according to Fisher’s protected LSD test ($p = 0.05$). Vertical bars represent mean ± S.D. ($n = 5$).
Figure 2-3. Effect of IN937b on squash root and shoot dry weight. A, Average dry weight of squash shoots measured 30 days after emergence. B, Average dry weight of squash roots measured 30 days after emergence. In the fertilization experiment, plants were fertilized with water soluble fertilizer (20:20:20, 3.5 g/mL, 20 mL/plant) once each week. Values with same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05). Vertical bars represent mean ± S.D. (n = 10).
Figure 2-4. Chlorophyll content in leaves of squash plants treated with PGPR. PGPR strains were applied twice as a soil drench to squash roots. The concentration of IN937b, SE76, and IN937a was adjusted to $10^8$ CFU/mL, and 20 mL was applied to each plant. Actigard® (30 mg/L; 20mL/plant) was applied as a positive SAR control of Phytophthora blight. The SPAD measurement were carried out 5 days after inoculation. SPAD values were measured on three leaves at same position on each plant. Values with same letter within a column are not significantly different according to Fisher’s protected LSD test ($p = 0.05$). Vertical bars represent mean ± S.D. ($n = 5$).
CHAPTER 3
THE ROLE OF BIOFILM FORMATION AND COLONIZATION IN THE RHIZOSPHERE
BY BACILLUS SUBTILIS IN937B WITH SYNTHESIS OF SURFACTINS

Introduction

Plants attract beneficial rhizobacteria such as PGPR by exudates released from plant roots (Raaijmakers et al., 2009b). PGPR are directly involved in the nutrition, health and quality of plants. They have a role in cycling carbon, nitrogen, and phosphorus, as well as suppressing soil-borne plant pathogens (Garbeva et al., 2004). The PGPR-mediated nutrient cycling is related to increase nitrogen-fixation and the availability of soluble phosphates in the soil. The mechanism by biocontrol-PGPR protects plant roots from pathogens include microbial antagonism, biofilm formation and the stimulation of induced systemic resistance in the host plants. The functional activities and community diversities of PGPR are positively affected by plant roots. Roots are able to release a wide range of organic and inorganic compounds into the rhizosphere including sugars, amino acids, phenolics, enzymes, siderophores, etc. (Pinton et al., 2001). The root exudates create specific habitat conditions suitable for microbial development, and play an important role in affecting microbial populations, availability of nutrients,

PGPR colonize the plant roots steadily by chemotaxis attraction and biofilm formation (Yaryura et al., 2008). The process includes bacterial attachment to the root, movement and proliferation of PGPR along the elongating root, and penetration of the epidermis (Liu et al., 2011). PGPR are likely to locate roots through particular signals emanating from the roots. Carbohydrates and amino acids also stimulate PGPR to move towards roots (Bais et al., 2006). PGPR can also produce biofilms to initiate beneficial interactions with host plants. Biofilm foster increased resistance to certain
environmental stresses as well as antimicrobial tolerance (Danhorn and Fuqua, 2007). Biofilm is a multicellular structure which adheres to the root surface as a protective mechanism against adverse environmental conditions. The formation of biofilm is associated with root colonization by bacteria such as PGPR (Ahmad et al., 2011).

Root colonization by PGPR is considered integral for PGPR to be successful as biocontrol agents (Whipps, 2001). The biocontrol effect depends on population densities of PGPR applied for suppressing pathogens (Kumar et al., 2011a). For example, *P. fluorescens* Pf1TZ could protect grape vine (*Vitis vinifera* L. cv. Chardonmy) plantlets against *Botrytis cinerea* after 3 weeks of bacterial inoculation (Kilani-Feki et al., 2010).

The population density of Pf1TZ colonized roots required for the bacteria to protect plants from the pathogen was $10^4$ CFU/mg of fresh roots. However, shortly after PGPR inoculation, the population declines progressively over time (vanVeen et al., 1997). Natural complexity of the rhizosphere (soil pH, temperature, nutrients, competitors, and predators) poses challenges to PGPR survival in different soil environments (Strigul and Kravchenko, 2006). As effective biocontrol agents, PGPR must establish themselves in the rhizosphere, which is affected by many abiotic and biotic factors.

*Bacillus* PGPR strains are widely considered to be capable of colonizing roots (Compant et al., 2010). For example, *B. pumilus* SE34 grew on the root surface and intercellularly penetrated roots of pea plants, allowing *B. pumilus* SE34 to get nutrients from the plant and compete for root colonization (Benhamou et al., 1996). Many *Bacillus* strains are capable of adopting and switching between free-living (planktonic) and biofilm (surface-attached) forms. The planktonic form can rapidly proliferate as free-floating individual cells, whereas the biofilm form are a unit of well-organized bacterial
cells which helps to tolerate adverse environments (O’Toole et al., 2000). As highly structured microbial communities, biofilm helps bacterial strains adhere to a solid surface where nutrients in an aqueous environment tent to concentrate. It is a survival strategy that designed to anchor PGPR in a nutritionally advantageous environment and permit their escape when growth factors have been exhausted.

*Bacillus* strains have the ability to form biofilm both in the laboratory and in nature (O’Toole et al., 2000). Under laboratory conditions, *B. subtilis* 3610 produced architecturally complex colonies on solid medium and formed floating biofilm called pellicles at the interface of the liquid medium and air (Lopez et al., 2009). *Bacillus subtilis* 3610 forms robust biofilms on the root surfaces of tomato plants (Chen et al., 2013). Biofilm formation by *Bacillus* plays an important role in protecting plants from pathogen attacks. *B. subtilis* ATCC 6051 suppress *Pseudomonas syringae* on *Arabidopsis* by competition on colonization (Bais et al., 2004). *Paenibacillus polymyxa* B1 and B2 protect *Arabidopsis thaliana* from infections by *Pythium aphanidermatum* by biofilm formation on root tips (Timmusk et al., 2005).

Biofilm formation by *Bacillus* spp. is associated with production of surfactins, which are well-known secondary metabolites with antifungal activities. Surfactins produced by *Bacillus* spp. are required for biofilm formation and for the successful biocontrol of plant pathogens. A knock-out mutant of surfactin synthase resulted in the loss of swarming motility of *B. subtilis* which involves the differentiation of vegetative cells and population migration across solid root surfaces (Kinsinger et al., 2003; Koumoutsi et al., 2004). Mutants deficient in surfactin synthesis were unable to suppress pathogens (Bais et al., 2004b). *B. amyloliquefaciens* FZB 42 produces surfactins. Knock-out
mutants of surfactin production were unable to repress *Fusarium oxysporum* grown on agar plates (Koumoutsi et al., 2004). Moreover, surfactins obtained from *B. amyloliquefaciens* strain S499 were capable of triggering an oxidative burst which is mediated perception by tobacco root cells that induce a defensive state in the plant (Henry et al., 2011). These results indicate that surfactins play an important role in root colonization by *Bacillus* spp. and in disease suppression via direct inhibition and stimulation of the host plant’s immune system (Ongena and Jacques, 2008).

The possible role that surfactins play in biofilm formation includes functioning as bio-surfactants, which have hydrophobic properties for water-insoluble substrates (Ron and Rosenberg, 2001), and influences on attachment and detachment of microorganisms to roots (Branda et al., 2001). *Bacillus subtilis* has long served as the powerful model organism for laboratory studies the mechanisms of biofilm formation (Vlamakis et al., 2013). However, studies need to be done to expand our understanding of *Bacillus* PGPR biofilm formation on plant roots, or whether biofilm influences root colonization by *Bacillus* PGPR.

In the present study, we focused on *in vitro* assays of biofilm formation and colonization of selected *Bacillus* PGPR strains in the rhizosphere of squash. The selected strains of *Bacillus* PGPR were able to suppress Phytophthora blight disease caused by *P. capsici* through soil inoculation (see Chapter 2). The aim of this work were the following: (1) to evaluate the ability of *Bacillus* PGPR to form biofilm *in vitro*, (2) to investigate the timing and concentration of *B. subtilis* IN937b required for biofilm formation, (3) to determine biosynthesis of surfactin characteristics of PGPR strains.
corresponding to biofilm formation, (4) to assess whether IN937b would colonize and form biofilm on squash roots.

Materials and Methods

PGPR Strains and Media

All *Bacillus* PGPR strains used in this study were stored in nutrient broth (NB) containing 15% glycerol at -80 °C prior to use. Bacterial cultures were prepared first by streaking each PGPR strain taken from ultra-cold storage onto nutrient agar (NA; Difco) plates, then incubating them at 28 °C for 24 h.

For pellicle formation experiments, a minimal medium MSgg (5 mM potassium phosphate, pH 7, 100 mM MOPS, pH 7, 2 mM MgCl₂, 700 μM CaCl₂, 50 μM MnCl₂, 50 μM FeCl₃, 1 μM ZnCl₂, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg ml⁻¹ tryptophan, 50 μg ml⁻¹ phenylalanine and 50 μg ml⁻¹ threonine) was used (Branda et al., 2001).

Surfactin production by PGPR was elicited using No. 3S medium containing 10 g of soybean peptone, 10 g of glucose, 1 g of KH₂PO₄, and 0.5 g of MgSO₄·7H₂O per liter of the medium (pH 7.0) (Tsuge et al., 2005).

Bacteria were grown in Luria broth (LB) medium for DNA extraction: 10 g of bacto tryptone (Difco), 5 g of yeast extract (Difco), 10 g of NaCl per liter of the medium (pH 7.0) (Gellert et al., 1976).

Pellicle Formation

PGPR strains were grown in 5 mL Nutrient Broth (NB; Difco) medium on a rotary shaker incubator at 150 rpm overnight at 28 °C. Five microliters of cell cultures were transferred into 1 mL liquid MSgg medium in 10 mL plastic tubes (or 30 mL liquid MSgg medium in 100 mL beakers). The plastic tubes and beakers were placed in the
incubator at 28 °C for 3 days when the pellicle, a biofilm formed at the air-liquid interface, was observed and photographed with a Canon EOS Rebel T1i digital camera (Canon, Tokyo, Japan).

**Colony Morphology Assay**

For colony architecture analysis on the solid media, PGPR strains were streaked on NA plates to get the single colonies. The plates were incubated at 28 °C for at least 24 h to observe morphology of the single colonies. The architecture was clearly observable under a dissecting microscope (Olympus DF Planapo IX, Olympus, Tokoya, Japan). Colonies were imaged using a Canon EOS Rebel T1i digital camera.

**Biofilm Production Assay in Microtiter Plates**

*Bacillus* PGPR strains were grown in 10 mL NB medium at 28 °C on a rotary shaker incubator at 150 rpm overnight. Biofilm production assays were performed with MSgg medium (Branda et al., 2001). The inoculum for the microtiter plate assays was obtained by diluting the *Bacillus* cells to $10^6$ CFU/mL. Final inocula were diluted to an identical density and calculated based on OD$_{600}$. One hundred microliters of the inoculum were grown in each well of the 6-well polyvinylchloride (PVC) microtiter plates, each containing 10 mL liquid MSgg medium. The bacterial cells were grown at room temperature for 7 days when they reached the stationary growth phase. Biofilm production and morphologies were imaged using a Canon EOS Rebel T1i digital camera.

**DNA Extraction and Polymerase chain reaction (PCR) Analysis**

*Bacillus* strains were inoculated into 10 mL LB medium and incubated on a rotary shaker incubator at 150 rpm overnight at 28 °C. DNA extraction was carried out using
the Qiagen kit (Qiagen QIAamp DNA mini kit, Qiagen, Valencia, CA) according to the manufacturer’s instructions. Polymerase chain reaction (PCR) reactions were carried out based on the methodology and specific primers established by Hsie et al. (2004). Primers used are listed in Table 3-1, that amplify genes encoding the antibiotics synthetases of iturin A, bacillomycin D, surfactin, zwittermicin A, and fengycin (Hsieh et al., 2004; Ramarathnam et al., 2007).

The PCR reaction was performed in a final volume of 25 µL containing 2.5 µL of 10X PCR buffer, 0.5 µL of dNTP mix, 1.0 µL each of PCR primers, 0.5 µL of Taq DNA polymerases, and 1 µL each of template genomic DNA. Amplification was done with a conventional thermal cycler (BioRad, Laboratories, Hercules, CA) with an initial denaturing at 94 °C for 1 min, annealing at 46 °C for 30s and extension at 72 °C for 1 min, for a total of 30 cycles, and followed by a final extension of 72 °C for 10 min. Following amplification, the PCR reaction mix was separated through 1% agarose gel electrophoresis.

PCR products were purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA). DNA sequencing was conducted at the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL. Sequence data were processed and analyzed with Basic Logal Alignment Search Tool (BLAST) software of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST). The amino acid sequence of sfp in IN937b was determined using the translation tool in the software DNAMAN version 7 (Lynnon Biosoft, Quebec, Canada). The amino acid sequences for *B. amyloliquefaciens* FZB42, *B. cereus* E33L, *B. megaterium* QM B1551, *B. polymyxa* SC2, *B. pumilus* ATCC 7061,
B. subtilis 168, B. subtilis IN937b, Brevibacillus brevis NBRC 100599, and B. licheniformis ATCC14580 were obtained from GenBank (http://www.ncbi.nlm.nih.gov/).

Multiple sequence alignment and Phylogenetic analysis were done with DNAMAN version 7. Phylogenetic analysis was performed using a non-rooted neighbor-joining tree with Poisson-correction (Bootstrap values: 1000 replicates) distances.

**Surfactin Extraction**

Surfactin was extracted from PGPR cultures according to Yakimov et al. (1995). Bacterial cells were grown in No. 3S medium on a rotary shaker incubator at 150 rpm for 48h at 28 °C. Cells were separated from No.3S medium by centrifugation at 3,000 rpm for 30 min at room temperature. The supernatant was subjected to acid precipitation by adding 6 M HCl to achieve a final pH of 2.0 and allowing the precipitate to form at 4 °C overnight (Yakimov et al., 1995). The pellet was collected by centrifugation at 3,000 rpm for 30 min at room temperature, and washed two times with DI water (pH 2.0, adjusted by 6M HCl). The pellet was dissolved in methanol (pH 7.0, adjusted by 1 M NaOH) by stirring for 2 h, and was placed in the refrigerator at 4 °C overnight. The crude material was collected for further analyses.

**HPLC/ESI-MS\textsuperscript{n} Assay of Surfactin Production**

The crude material extracted as described above and the surfactin standard (Sigma-Aldrich, St. Louis, USA) were sent to Dr. Jodie V. Johnson’s laboratory in the Chemistry Department at the University of Florida, Gainesville, FL for detecting the presence of surfactins and for further quantitative analyses. The extracts and surfactin standard were analyzed via high performance liquid chromatography combined with electrospray ionization tandem mass spectrometry (HPLC/ESI-MS\textsuperscript{n}).
Both (+) and (-) ESI-MS\textsuperscript{n} collision-induced dissociations (CID) were conducted to quantify surfactins produced in the testing samples. Both positive and negative ion modes are provided in the Table 3-4. The gradient was changed to allow better separation in (+)ESI-MS compared to (-)ESI-MS. (+)ESI-MS was chosen for further analyses. The relative amount of different surfactin isoforms was detected in the crude extracts of IN937b expressed in terms of HPLC/(+)ESI-MS peak areas and relative areas. The sum of the intensities of the [M+H]+ and [M+Na]+ were used to produce the ion-peaks for the surfactins. The peak area was obtained by integration of the appropriate peak in the relative abundance of surfactins in the ion chromatograms (Table 3-5).

**Screening IN937b Mutant for Rifampicin Resistance**

Spontaneous mutants with rifampicin-resistance were selected from nutrient agar amended with rifampicin (NAR) plates according to Bolstridge et al. (2009) with modifications. Wild type of *B. subtilis* IN937b was plated on NA amended with 10 µg/mL rifampicin (NAR) and incubated at 28 °C for 48 h. The mutants with the same morphology as the wild type were transferred to NAR with the higher concentration of rifampicin (25 µg/mL). And the above step was repeated until the mutants had the ability to resist 75 µg/mL rifampicin. Stability of the rifampicin resistance was confirmed by sub-culturing ten times on NA and then on NAR (75 µg/mL). All mutants were stored in 20% glycerol at -80 °C (Bolstridge et al., 2009).

**Root Treatment by IN937b Rifampicin-Resistance Mutant**

A spontaneous mutant of IN937b resistant to 75 µg/mL rifampicin was used to monitor changes in the IN937b population introduced into the rhizosphere. A single colony of IN937b with rifampicin resistance was inoculated in 10 mL NB containing 50
µg/mL rifampicin in 50 mL centrifuge tubes. After the tube was incubated at 28 °C on a rotary shaker incubator at 150 rpm overnight, 1 mL of the bacterial suspension was inoculated into 200 mL NB in 500 mL flasks, and incubated for 24 h under the same conditions as mentioned above. The cell pellet was collected by centrifugation at 3000 rpm for 15 min and washed twice with sterile DI water. The cell concentration used in this experiment was adjusted to 10⁸ CFU/mL, determined by serial dilutions and plating.

Squash seeds were planted in plastic pots (7.62 cm high and 10.16 cm at the opening with 8 drain holes) filled with potting mix. One seed was placed in each pot. In the greenhouse, two-week-old squash plants were treated with rifampicin resistant IN937b by soil drench at 10⁷ CFU/g soil per plant.

**Rhizosphere Sampling**

Samples of the squash rhizosphere were taken from each of the pots 1, 3, 5, 7, and 14 days after IN937b treatment. The roots of the squash plants were shaken vigorously to get rid of bulk soil that did not tightly adhere to the roots. Four replicates in each treatment (IN937b and non-treated control) were obtained at each sampling time. The rhizosphere soil is defined as the soil still adhering to the roots after vigorous shaking. The roots and rhizosphere soil were collected together, and 50 g of soil and the roots were placed in a 500 mL plastic container with 100 mL sterile DI water. Total bacterial populations were assessed by serial dilutions and plating of the resulting soil suspension on NAR medium after the container was shaken on a rotary shaker incubator at 150 rpm for 30 min at room temperature.

**Recovery of IN937b Mutant with Rifampicin-Resistance from Roots**

For measuring internal root colonization, roots were separated from the rhizosphere samples mentioned above and were collected by rinsing the roots three
times with DI water. Root samples were surface disinfected with sodium hypochlorite (1%) for 5 min followed by ethanol (70%) for 30s. Then the roots were chopped and ground with 2 mL sterile DI water in a sterile mortar using a pestle. In order to make sure that no surface bacteria remained after surface-disinfection, one intact root for each sample before chopping was placed on the NAR medium for surface bacteria growing. The population of inoculated bacteria in internal root tissues was determined by serial dilutions and plating of the homogenate on the NAR plates and incubated at 28 °C for 48 h.

**Results**

**Architecture of Pellicles and Colonies of *Bacillus* spp.**

Tubes made of PVC which stimulate biofilm formation (Pedersen, 1990), were used to test if selected *Bacillus* PGPR had the ability to form biofilm on an abiotic surface. The *Bacillus* strains were allowed to grow on the surface of the liquid MSgg medium. Five days after inoculation, the biofilm was observed as a flat layer at the interface between the air and liquid medium (Figure 3-1 A.). PGPR strains IN937b, 1216S, GB03, IN937a, INR7 and 1PC-19 had the ability to form a thick, floating biofilm composed of a large group of bacterial cells. However, strains SE76, 1PC-11, SE34, SE49, SE52 and T4 exhibited poor growth of the layer, and they were unable to form a biofilm initially.

When spotted on NA plates, the *Bacillus* strains produced colonies with morphological characteristics of their corresponding pellicles (Figure 3-1 B.). Colonies varied widely in morphology among *Bacillus* strains. Five days after inoculation, PGPR strains IN937b, 1216S, GB03, IN937a, INR7 and 1PC-19 had wrinkled (because of its rough appearance) colonies which were consistent with the result of pellicle formation in
the liquid medium test (Figure 3-1 A.). SE76, 1PC-11, SE34, SE49, SE52 and T4 exhibited smooth (because of its small and flat appearance) colonies on NA plates which were not able to form biofilm in the liquid medium. The wrinkled bacterial colonies grew wider on the solid medium surface than the smooth colonies did.

Closer examination of the colonies under a dissecting microscopy (×10 magnification) revealed a highly structured bacterial community, with clearly observable architecture. As shown in Figure 3-2, the wrinkled pattern of IPC-19 generated denser layers compared to the other test PGPR strains. IN937b and 1216S consisted of many wave-like columns that projected upward from the agar surface. IN937a, INR7 and GB03 had aerial structures composed of cells bundled together in a flat form.

**In Vitro Test for Biofilm formation**

The highly structured biofilm was formed by the *Bacillus* strains on the surface of a liquid MSgg medium in a beaker. *Bacillus* PGPR strains SE34, SE52, SE76, and T4 failed to form biofilm (Figure 3-3). GB03 and 1216S formed an extremely thin, fragile, and smooth pellicle that lacked a distinctive architecture. Contrastingly, PGPR strains IN937b and 1PC-19 formed a thick pellicle with an intricate vein-like appearance on its surface, and a thick and highly folded structure of biofilm was clearly visible through the beaker.

**Effect of Bacterial Density on Biofilm Formation**

Based on the observation of the *in vitro* test for biofilm formation, wherein bacterial cells move in the liquid without a solid surface, we found that the process of self-organization in biofilm has been linked to bacterial density and bacterial growth speed in the liquid-air interface. In order to determine the influence of PGPR densities on biofilm formation, we assayed the selected PGPR strains in liquid MSgg medium in
polystyrene plates that has 6 wells each. Biofilm formation and progression were visually inspected daily for 7 days. The initial concentration in liquid MSgg medium was calculated as $10^4$, $10^3$, and $10^2$ CFU/mL. At days 1 and 2 after bacterial inoculation, IN937b had a high concentration ($10^4$ CFU/mL) and the positive strain 1PC-19 ($10^4$ CFU/mL) formed thin, loosely associated aggregations of bacterial cells at the surface of the liquid medium in the well. At days 2 and 3, a thin pellicle comprised of both live cells and extracellular materials formed on the air-surface interface in the wells containing IN937b at $10^4$ and $10^3$ CFU/mL and 1PC-19 (Figure 3-4). From days 3 to 5, the pellicle at the interface thickened and developed a thick mat of cording and reticulations. In addition to bacterial cells, extracellular materials from biofilms produced by the strain 1PC-19 adhered to and moved up the walls of the well during days 2 to 5 (Figure 3-4 and Table 3-2). IN937b at the lowest concentration ($10^2$ CFU/mL) and the negative strain SE52 failed to form biofilm and the reticulation was incomplete, bacterial cells were unable to disperse in the well and could not self-organize.

**PCR Detection of Lipopeptide Genes in Bacillus PGPR Strains in Relation to Biofilm Formation**

A 675-bp PCR product was amplified from IN937b using the primers specific to the *sfp* gene which is responsible for surfactin production (Figure 3-8). Amplification of the PCR products indicates that IN937b and IN937a harbor the *sfp* gene and may produce surfactins. No PCR product was amplified from SE76 and SE52 using any of the primers tested. No product was amplified from IN937b with primers specific to the genes encoding the antibiotics iturin A, fengycin, zwittermicin A, and bacillomycin D (Table 3-3).
DNA sequences analyzed by BLAST software revealed that this PCR fragment in IN937b had 100% similarity to *B. subtilis* 168 (Accession No. AL009126) whose genome has been fully sequenced. The results indicated that IN937b is positive for the presence of the 4'-phosphopantetheinyl transferase gene *sfp* for surfactin synthesis. Alignment of the predicted amino acid sequences revealed that all the 5 amino acid residues are conserved in all *Bacillus* for surfactin synthetase (Figure 3-9). The phylogenetic tree based on amino acid sequences of *sfp* revealed a close relationship among *B. subtilis* IN937b, *B. subtilis* 168, and *B. amyloliquefaciens* FZB42 (Figure 3-10).

**HPLC/MS Analysis of Surfactins Production in *B. subtilis* IN937b**

The methanol extracts of No. 3S culture of strain IN937b were separated by HPLC/ESI-MS, and a clear peak pattern of surfactins was eluted based on the molecular weight range of the standard sample. The molecular weight (MW) of surfactin isoforms was determined by ESI-MS (Figure 3-5 A-G), and the HPLC fractions that had more than one peak were found to represent a cluster of isoforms at MW 993, 1007, 1021, 1035, 1049, and 1063 Da. The fatty acids in surfactins were determined (Table 3-4). The structure of standard surfactins consists of a seven amino acid cyclopeptide with a saturated fatty acid chain (R) attached. The chain length of R varies from C₉ to C₁₄ leads to detection of the major molecular weights. Surfactin isoforms with MW 1035 and 1021 Da contained the highest levels of relative abundance of 45.76% and 39.84%, respectively (Table 3-5).

**Survival of IN937b in the Rhizosphere**

Recovery of IN937b from the rhizosphere began at 1 day after treatment to two weeks after soil drench with IN937b (Figure 3-6). There was a high bacterial density at
10^6 CFU/g soil per plant in the potting mix at 1 day after application. The population declined dramatically to 10^4 CFU/g soil per plant at 5 days after treatment. And then the populations slowly decreased to 10^3 CFU/g soil per plant at 14 days after treatment.

**Survival of IN937b in Squash Roots**

Internal root colonization by IN937b was evaluated by serial dilutions and plating on rifampicin-amended NA plates. No IN937b cells were detected on plates from the non-treated squash roots after surface-sterilization. IN937b was recovered in squash roots at 1 day after treatment. However, the endophytic population of IN937b recovered from the roots fluctuated from 10^1 to 10^3 CFU/g fresh root (Figure 3-7). At 2 weeks after IN937b treatment, an increasing trend in the population of IN937b was observed with level up to 10^3 CFU/g fresh root. Bacterial densities of IN937b colonized in the roots were as high as 10^3 CFU/g fresh root 14 days after treatment.

IN937b colonized the rhizosphere and also occupied the internal tissues of squash roots after application. At the early stage of colonization, the population of IN937b survived in the rhizosphere. Over a 14 days period, the bacterial population in the rhizosphere decreased, while endophytic population in root tissue survived longer and accumulated gradually.

**Discussion**

*Bacillus* strains had the ability to form biofilms in liquid or solid medium. Roots of some plants contain tightly bound *Bacillus* strains that form abundant biofilms. Both *in vitro* and *in vivo* assays have showed that the rapid formation of biofilm under laboratory conditions reflect the ability of biofilm formation in the natural environment (Bais et al., 2004b). Interestingly, all strains which morphologically vary in colony morphology, are
able to form biofilms, implying that *Bacillus* strains developed the same major adaptive strategy to survive in the soil environment.

Under laboratory conditions, the bacterial cells produced extracellular polymers that bind cells together and provide structural support for biofilm that resided at the liquid-air interface of the medium in glass beakers. The lack of solid surface at this interface imposes a greater requirement for self-organization on the constituent cells. When grown in beakers filled partly with a liquid medium, bacterial strains form large groups of cells on the liquid surface. During this process, bacterial strains produce extracellular polysaccharide (EPS), which affords better access to oxygen (Flemming and Wingender, 2010). EPS is effective in binding cells together and in anchoring them to the beaker walls.

The architecture of the colony and the thickness of the pellicle formed at the air-liquid interface are both influenced by surfactin produced from different *Bacillus* strains (Leclere et al., 2006). Among these, *B. subtilis* is believed to form a robust biofilm when it attains a certain population density. The motile cells of *B. subtilis* migrate to the air-medium interface and biofilm formation rate is associated with cell populations. *B. subtilis* cells assimilate sufficient oxygen to produce submerged fermentation that is subjected to colony morphology and growth rate. Faster growth of bacterial cells causes nutrient depletion in the culture, resulting in faster sporulation of *Bacillus* spp. Sporulation of *B. subtilis* is delayed in biofilm when compared to that of *B. subtilis* at submerged fermentation (Lindsay et al., 2005). Therefore, *Bacillus* cells in the biofilm remain in their metabolically active state for a longer period of time compared to those in their sporulation stage.
Bacillus subtilis often found in the rhizosphere might be closely associated with plant roots. Recently, the ability of Bacillus in the rhizosphere to form beneficial biofilm has been well documented (O'Toole et al., 2000; Vlamakis et al., 2013). The behavior of bacterial cells tending to attach and aggregate near plant root surfaces or internal roots by self-organization can be considered a type of biofilm (Lugtenberg et al., 2001). These communities have spatial and temporal organization due to cellular differentiation (Ramey et al., 2004).

Biofilm helps microbial populations to swarm over the root surface and reach new niches to attain more nutrients (Branda et al., 2001). On the well-colonized root surface, approximately 10 - 40% of root surfaces can be occupied by Bacillus that formed biofilm (Vlamakis et al., 2013). Motility of the bacterial cells is important for biofilm formation and colonization by the bacteria. The motility that performed as swimming and swarming was achieved through the cell movement of colonies. The strains with fast motility usually spread or occupy large area in the medium, indicating that motility is an important mechanism for Bacillus colonization, especially for those introduced to a new environment (Kinsinger et al., 2003). As a first step of biofilm formation, the motility that helps bacterial strains to access favorable colonization sites in the rhizosphere involves rapid and coordinated population migration. As a second step, bacterial cells must attach to highly structured biofilms on solid surfaces or pellicles and adhere to roots and soil particle surfaces (Danhorn and Fuqua, 2007).

The population of IN937b recovered from potting mix was $10^3$ CFU/g of fresh root 10 days after IN937b introduced into the rhizosphere of squash plants. The results from the survival assay of IN937b showed poor colonization in the rhizosphere, although
IN937b formed robust biofilms in defined medium (and likely on plant roots as well). Also, results from the microtiter assays showed that biofilm formation strongly depended on the initial population of IN937b inoculated in the medium, indicating that initial population might be critical for biofilm formation.

In this experiment, the initial populations of IN937b were high and dramatically decreased in the potting mix 2 weeks after treatment. Potting mix is a relatively simple environment compared to the soil, which is a mixture of mineral compounds and microorganisms. Bacterial behavior may be influenced by environmental factors including the nutrients, water content in the soil and bacterial movement in the root systems. On the other hand, the rapid colonization of IN937b in the internal roots indicated that IN937b had potential to predominate in the root competition. These also enable *Bacillus* strains to find colonization sites and acquire nutrients for multiplication over a long period.

Different types of distribution were found in *Bacillus* strains. Some bacteria were localized at the root cap while others were evenly distributed along the root surface. Fluorescence *in situ* hybridization (FISH) revealed that the favorable site for *Bacillus* strains to form biofilm could be extended 2-30 µm in the rhizosphere outward from root surface of wheat (Watt et al., 2006). Effective colonization leads to plant growth promotion (Ongena et al., 2005). *Bacillus* strains secrete a large number of compounds into the rhizosphere which are beneficial to plant roots and shoot growth. *Bacillus* works as biofertilizer for many plant species due to its interaction with plant roots through biofilms on root surfaces (Seneviratne et al., 2010).
Bacillus strains in this study were selected from different crops in diverse environmental niches. These bacteria showed distinct features, especially in colony morphology, biofilm formation and biocontrol activity. However, the ability to form biofilms is still highly conserved in these test strains. These results imply that different Bacillus spp. have developed the same strategy for adapting to different environments (Chen et al., 2013).

*B. subtilis* IN937b produced surfactins. Surfactins are well known to stimulate biofilm formation (Bais et al., 2004a). For example, surfactins are detected in the rhizosphere of cucumber and tomato inoculated with *B. subtilis* strains (Ongena et al., 2007; Kinsella et al., 2009). The movement of bacterial cells is largely dependent on the production of surfactins, which help cells to spread over the root surfaces (Raaijmakers et al., 2006). Surfactin production is widespread in Bacillus genus (Price et al., 2007). Surfactin-producing *Bacillus* species can be easily isolated from soils. However, surfactins are susceptible to degradation by other microorganisms in nature or in the laboratory. In sterile soils without interference from other microorganisms, levels of surfactins remained stable for 25 days (Asaka and Shoda, 1996).

Surfactins are important for microorganisms because they lower the surface tension of water. In addition, surfactins have antimicrobial activities due to their abilities to penetrate cellular membranes (Heerklotz and Seelig, 2007). Moreover, surfactins act as a signal in the quorum-sensing to regulate bacterial population density in response to environmental changes (Lopez et al., 2009). Surfactins are reported to act as a stimulus in biofilm formation. High levels of surfactins were observed during colonization and biofilm formation by *B. amyloliquefaciens* S499 on tomato roots. Recovery of
Surfactins from the rhizosphere indicated that bacterial colonization on the root leads to high levels of surfactins that stimulate biofilm formation (Nihorimbere et al., 2012).

Surfactin biosynthesis requires enzymes called surfactin synthetases. The gene \textit{sfp} serves a regulatory role as well as a more direct role in surfactin synthesis. In this study, PCR primers specific to the regions within \textit{sfp} were used to screen a number of \textit{Bacillus} spp. strains for presence of the \textit{sfp}. Only IN937b was confirmed to carry the \textit{sfp} gene responsible for the production of surfactins.

To confirm the PCR result for surfactin production, HPLC and quantitative analysis were adopted to detect the amount of surfactins produced from \textit{B. subtilis} strain IN937b. Surfactins were extracted from No. 3S culture broth of IN937b by classic methods including acid precipitation with HCl, recrystallization and extraction by organic solvents such as methanol. Results of HPLC were consistent with the PCR detection.

Naturally occurring surfactins are a mixture of different molecules based on chain length, β-hydroxy fatty acid, and amino-acid sequence (Lin and Jiang, 1997). In the present study, several isomers of surfactins with different molecular weights were detected.

Biofilm is important for bacteria to colonize in the rhizosphere and to act as a biocontrol agent (Bais et al., 2004b). The biocontrol efficacy of \textit{B. subtilis} against \textit{Rhizoctonia solani} has been shown to be achieved by the production of surfactins and iturin A. It is possible that biofilm and surfactin production allowed \textit{B. subtilis} and its close relatives, such as \textit{B. amyloliquefaciens}, to efficiently colonize plant roots and also to provide protection to their hosts. A knockout of the \textit{sfp} gene resulted in flat and small colonies of the bacterium. This knockout mutant spread very little on the agar surface.
(Branda et al., 2001) revealing that the mutant failed to form mature aerial structures related to biofilm formation. Loss of surfactin biosynthesis led to a deficiency in motility and reduced colonization of roots and surfaces. Biocontrol of *P. syringae* infection of *Arabidopsis* roots by *B. subtilis* 6051 is related to surfactin production on the surface of the root (Bais et al., 2004b). *B. subtilis* 6051 can produce a variety of lipopeptides including surfactins that are important for maintaining the aerial structure of biofilms. Upon root colonization, *B. subtilis* 6051 formed a stable, extensive biofilm and secreted surfactins, which act together to protect plants against infection by pathogenic bacteria (Morikawa, 2006). It is strongly suggested that the production of surfactins is essential for biofilm formation and colonization of plant roots.

In conclusion, our results suggest that many *Bacillus* PGPR strains are capable of forming biofilms. It will be important to construct knock-out mutants of surfactin synthesis genes. Future work is needed to determine the physiological relevance of the regulation of surfactin synthesis genes and biofilm formation. The surfactin mutants will continue to be useful in future studies on colonization models in the squash rhizosphere, and this capability may be important for disease suppression. Biofilm formation by IN937b may increase its colonization to the squash root. However, the exact mechanisms in which biofilm formation contributes to plant biocontrol still remain unknown. Successful colonization on root tissue is a first step critical in eliciting plant biocontrol activities against soil borne pathogens. In the future, it will be imperative to characterize the signaling molecules released by the plant host that improve *Bacillus* spp. ability to colonize and form biofilm on the root surfaces.
Table 3.1. List of primer sequences used for biosynthetic genes of surfactins, bacillomycin, iturin A, Fengycin, and Zwittermicin A.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Primer sequence</th>
<th>(Hsieh et al., 2004)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surfactin</td>
<td>F: ATGAAGATTTCAGGAATTTA R: TTATAAAAGCTCTTCCTACG</td>
<td></td>
</tr>
<tr>
<td>Bacillomycin D</td>
<td>F: GAAGGACACGGGCAGAGAGTC R: CGCTGATGACTGTCATGCT</td>
<td>(Athukorala et al., 2009)</td>
</tr>
<tr>
<td>Iturin A</td>
<td>F: GATGCGATCTCCTTGGATGT R: ATCGCATGTGCTGCTTGAG</td>
<td></td>
</tr>
<tr>
<td>Fengycin</td>
<td>F: TTTGGCACGAGGACAGTTC R: GCTGTCCGTCTGCTTTTC</td>
<td>(Ramarathnam et al., 2007)</td>
</tr>
<tr>
<td>Zwittermicin A</td>
<td>F: TTGGGAAGTTTACAGCTCT R: GACCTTTTGAAATGGGCCTA</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Biofilm formation by *Bacillus* strains with initial bacterial populations

<table>
<thead>
<tr>
<th>Concentration (CFU/mL)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Biofilm formation time (Days)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN937b (10⁴)</td>
<td>2</td>
</tr>
<tr>
<td>IN937b (10³)</td>
<td>3</td>
</tr>
<tr>
<td>IN937b (10²)</td>
<td>5</td>
</tr>
<tr>
<td>IN937b (10¹)</td>
<td>None</td>
</tr>
<tr>
<td>IN-19 (10⁴, positive strain)</td>
<td>3</td>
</tr>
<tr>
<td>SE52 (10⁴, negative strain)</td>
<td>None</td>
</tr>
</tbody>
</table>

<sup>a</sup> The inocula for the microtiter plate assays were obtained by diluting the *Bacillus* cells to approximately 10⁴, 10³, 10², and 10 CFU/mL. One hundred µL of inocula were grown in 6-well PVC microtiter plates containing 10 mL of liquid MSgg medium at room temperature for 5 days before imaging.

<sup>b</sup> Biofilm growth were observed by visual inspection. The biofilm formation was checked daily for the timing of biofilm formation.

Table 3.3. Detection of the genes related to biofilm formation

<table>
<thead>
<tr>
<th>Antibiotic&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IN937b</td>
</tr>
<tr>
<td>Surfactin</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Zwittermicin A</td>
<td>-</td>
</tr>
<tr>
<td>Bacillomycin D</td>
<td>-</td>
</tr>
<tr>
<td>Iturin A</td>
<td>-</td>
</tr>
<tr>
<td>Fengycin</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Five selected antibiotics are antimicrobial peptides that are associated with biosurfactins and in the fitness of *Bacillus* strains in the rhizosphere.

<sup>b</sup> Polymerase chain reaction (PCR) screening using specific primers (Table 3-1) for antibiotic biosynthetic genes in strains IN937b, SE76 and SE52. + represents detection of the genes. – represents no detection of the genes.
Table 3-4. Identification of surfactin ions by HPLC/ESI-MS

<table>
<thead>
<tr>
<th>R mass, u</th>
<th>MW, u</th>
<th>(+)ESI-MS</th>
<th>(-)ESI-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>C9H19 127.1</td>
<td>993.6</td>
<td>994.3</td>
<td>1016.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>992.5</td>
<td>1014.6</td>
</tr>
<tr>
<td>C10H21 141.2</td>
<td>1007.7</td>
<td>1008.4</td>
<td>1030.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1006.6</td>
<td>1028.7</td>
</tr>
<tr>
<td>C11H23 155.2</td>
<td>1021.7</td>
<td>1022.5</td>
<td>1044.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1020.7</td>
<td>1042.8</td>
</tr>
<tr>
<td>C12H25 169.2</td>
<td>1035.7</td>
<td>1036.4</td>
<td>1058.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1034.6</td>
<td>1056.7</td>
</tr>
<tr>
<td>C13H27 183.2</td>
<td>1049.7</td>
<td>1050.4</td>
<td>1072.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1048.6</td>
<td>1070.7</td>
</tr>
<tr>
<td>C14H29 197.2</td>
<td>1063.7</td>
<td>1064.5</td>
<td>1086.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1062.7</td>
<td>1084.8</td>
</tr>
</tbody>
</table>

The surfactin consists of a seven-amino acid cyclopeptide with a saturated fatty acid chain (R) attached. The chain length of R varies from C9 to C14 to provide the major molecular weights (u) detected. Mass R represents the molecular weight of saturated fatty acid chain. MW represents the molecular weight of surfactins.

HPLC-ESI-MS ions assay for surfactins. Both positive and negative ion modes were given in the table. (+)ESI-MS represents positive ion mode; (-)ESI-MS represents negative ion mode.

Table 3-5. HPLC/(+)ESI-MS peak areas and relative areas for the detected surfactins in the crude extracts of IN937b.

<table>
<thead>
<tr>
<th>MWa</th>
<th>Isomer 1</th>
<th>Isomer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-1minb</td>
<td>Area-1c</td>
</tr>
<tr>
<td>993</td>
<td>55.94</td>
<td>1,081,996,051</td>
</tr>
<tr>
<td>1007</td>
<td>56.91</td>
<td>1,966,233,219</td>
</tr>
<tr>
<td>1021</td>
<td>58.07</td>
<td>26,434,118,167</td>
</tr>
<tr>
<td>1035</td>
<td>58.80</td>
<td>30,383,407,593</td>
</tr>
<tr>
<td>1049</td>
<td>59.92</td>
<td>2,308,838,698</td>
</tr>
<tr>
<td>1063</td>
<td>60.60</td>
<td>601,132,399</td>
</tr>
<tr>
<td>SubTotal</td>
<td>62,775,726,127</td>
<td>3,625,713,362</td>
</tr>
<tr>
<td>Total</td>
<td>66,401,439,489</td>
<td>0.91</td>
</tr>
</tbody>
</table>

(+)ESI-MS mass chromatograms of the [M+Na]+ ions of the surfactins produced by IN937b, which match those of the surfactin standard.

RT represents retention time from 54 to 63 during which surfactins elute through the HPLC column.

The sum of the intensities of the [M+H]+ and [M+Na]+ were used to produce the ion-peaks for the surfactins. The peak area was integrated in the appropriate relative abundance ion chromatograms for determining the abundance of surfactins.
Figure 3-1. Architecture of pellicles and colony morphology of PGPR strains. A. Pellicle formation at the air-liquid interface in liquid MSgg medium of 12 *Bacillus* strains. Cells of *Bacillus* strains were inoculated in the liquid medium in the standing tubes at 28 °C for 3 days. The band at the top of the liquid medium represents the floating pellicle. B. Colony formation of 12 *Bacillus* strains on the surface of solid MSgg medium. Bacteria were streaked to form single colony on the medium and incubated at 28 °C for 24 hours.
Figure 3-2. Pellicle development of *Bacillus* strains. Pellicle formation at the air-liquid interface under a dissecting microscope (10×). Strains that formed biofilm in pellicle formation assays (Fig 3.1 A) were selected to observe pellicle morphology under a microscope. The floating pellicles at the top were carefully placed on top of a microscopic glass slide, and photographed (10×) for surface structure.

Figure 3-3. Biofilm development properties of *Bacillus* strains. Biofilm formation from selected *Bacillus* strains on the surface of a liquid MSgg medium in a beaker. Bacteria were inoculated in liquid MSgg medium in a beaker for floating pellicle development. The images were taken at room temperature 5 days after inoculation. All four strains (IN937b, IPC-19, 1216S, GB03) formed a biofilm with a wrinkled (rough surface appearance) pattern on the surface as shown in the top set of photo. The strains shown in the bottom set of photos were biofilm-negative phenotype.
Figure 3-4. Biofilm formation rate of *Bacillus* strains. Biofilm growth was observed by visual inspection. The inocula for the microtiter plates were obtained by diluting the *Bacillus* cells to approximately $10^4$, $10^3$, and $10^2$ CFU/mL. One hundred µL of inocula were grown in 6-well PVC microtiter plates containing 10 mL MSgg liquid medium at room temperature for 5 days before imaging. 1: IN937b (10^4 CFU/mL), 2: IN937b (10^3 CFU/mL), 3: IN937b (10^2 CFU/mL), 4: Control, 5: IPC-19 (+) (10^4 CFU/mL), 6: SE52 (10^4 CFU/mL) (-).
Figure 3-5. HPLC/MS analysis of the fraction from crude extraction from \textit{B. subtilis} IN937b culture.
Figure 3-5. Continued
Figure 3-5. Continued

E

F
A) Chromatogram of surfactins detected by HPLC with (+)ESI-MS. MW 1016, 1030, 1044, and 1058 with black underline, 1072 and 1086 are not shown in the figure. B) Surfactins with MW 993: HPLC/(+)ESI-MS/MS of m/z 1016 [M+Na]+: At least four isomers: two defined by m/z 707 and 772 and two defined by m/z 693 and 786, C) Surfactins with MW 1007: HPLC/(+)ESI-MS/MS of m/z 1030 [M+Na]+: At least four isomers: two defined by m/z 707 and 786 and two defined by m/z 693 and 800 product ions, D) Surfactins with MW 1021: HPLC/(+)ESI-MS/MS of the m/z 1044 [M+Na]+ ions shows the presence of at least four (4) isomers: m/z 707, 800, 693, and 814 product ions, E) Surfactins with MW 1035: HPLC/(+)ESI-MS/MS of m/z 1058 [M+Na]+: At least four isomers with two defined by m/z 707 and 814 and two defined by m/z 693 and 828, F) Surfactins with MW 1049: HPLC/(+)ESI-MS/MS of m/z 1072 [M+Na]+ ions shows more isomers: three defined by m/z 707 and 828, on the top, the m/z 707 shifted to m/z 721, G) Surfactins with MW 1063: HPLC/(+)ESI-MS/MS of m/z 1086 [M+Na]+ ions: The two major isomers were those defined by m/z 707 and 842 and on the bottom, the m/z 707 shifted to m/z 721.
Figure 3-6. Colonization of the rhizosphere of squash plants by a mutant of IN937b with rifampicin resistance. The data are expressed as log$_{10}$ CFU per gram of potting mix. Data points are the means and standard errors of four replicates. Data were collected at the 1, 3, 5, 7 and 14 days after treatment. Values with same letter within a column are not significantly different according to Fisher’s protected LSD test ($p = 0.05$). Vertical bars represent mean ± S.D. ($n = 5$).
Figure 3-7. Colonization of the squash roots by a mutant of IN937b with rifampicin resistance. The data are expressed as log_{10} CFU per gram of fresh squash root. Data points are the means and standard errors of four replicates. Data were collected at the 1, 3, 5, 7 and 14 days after treatment. Populations of IN937b colonizing squash seedling roots. Values with same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05). Vertical bars represent mean ± S.D. (n = 5).
Figure 3-8. PCR product profiles of the sfp gene. Gel displaying bands created by using primers for the sfp gene. Lane 1: Ladder 100-1000 bp makers, 2: SE76, 3: IN937a, 4: T4, 5: IN937b, 6: Negative control.
Figure 3-9. Comparison of the predicted amino acid sequence for the sfp gene present in several *Bacillus* strains. Multiple sequence alignment of sfp homologs from *B. amyloliquefaciens* FZB42 (FZB42), *B. cereus* E33L (E33L), *B. megaterium* QM B1551 (QMB1551), *B. polymyxa* SC2 (SC2), *B. pumilus* ATCC 7061 (ATCC7061), *B. subtilis* 168 (168), *B. subtilis* IN937b (IN937b), *Brevibacillus brevis* NBRc 100599 (NBRC100599), *B. licheniformis* ATCC14580 (ATCC14580). Conserved amino acid residues were shaded in the following manner: black = 100% identity or conserved substitution, grey ≥ 75% identity, light grey ≥ 50% identity, white < 50% identity.

Figure 3-10. Phylogram of sfp homologs from several *Bacillus* strains. Multiple sequence alignment of sfp homologs from *B. amyloliquefaciens* FZB42 (FZB42), *B. cereus* E33L (E33L), *B. megaterium* QM B1551 (QMB1551), *B. polymyxa* SC2 (SC2), *B. pumilus* ATCC 7061 (ATCC7061), *B. subtilis* 168 (168), *B. subtilis* IN937b (IN937b), *Brevibacillus brevis* NBRc 100599 (NBRC100599), *B. licheniformis* ATCC14580 (ATCC14580). An unrooted neighbor-joining phylogenetic tree using Poisson-correction (Bootstrap values: 1000 replications) distances was constructed based on the amino acid sequences alignment.
CHAPTER 4  
THE ROLE OF SUBTILOSIN A-LIKE ANTIMICROBIAL PEPTIDE IN SUPPRESSION OF PHYTOPHTHORA CAPSICI BY BACILLUS SUBTILIS IN937B

Introduction

*Bacillus* spp. are widely recognized as producers of secondary metabolites of low-molecular-weight compounds that are deleterious to other microorganisms (Thomashow et al., 2007). Approximately 4% of the *B. subtilis* 168 genome is devoted to the biosynthesis of secondary metabolites (Kunst et al., 1997). Similarly, approximately 8.5% of the whole genome in *B. amyloliquefaciens* FZB42 is dedicated to synthesize antibiotics which have significant potential for plant disease management (Chen et al., 2007).

The classification of antimicrobial metabolites produced by *Bacillus* spp. is largely based on non-ribosomal or ribosomal biosynthesis (Montesinos, 2007). Non-ribosomal peptide antibiotics are often modified posttranslationally, such as lipopeptides (surfactin, Chapter 3) and polyketides (Stein, 2005). This group of antibiotics possess antimicrobial capabilities, also involved in biofilm, swarming development, and induction of plant resistance (Ongena et al., 2005; Ongena et al., 2007). Secondary metabolites through ribosomal biosynthesis act as pheromones in quorum-sensing, or lead to programmed cell death. For example, bacteriocins have fungicidal and bactericidal capabilities for suppressing *Alternaria solani* and *Erwinia carotovora* (Hammami et al., 2012).

Antibiotic production by *Bacillus* spp. may play a major role in maintaining population density when they are introduced into the soil. The nutrition resources for microbial proliferation and survival are limited in the rhizosphere. Over time, consumption of the limiting nutrients will influence the dynamics between *Bacillus* and other microbes. *Bacillus* spp. compete with their neighbors for space and nutrients by
impairing or killing other microbes (Hibbing et al., 2010). As microbial growth inhibitors, antibiotics result in changes of microbial community structure by inhibiting growth of different species or related strains of the same species. In general, Bacillus spp. produce a wide range of antibiotics that are highly specialized for a given habitat may target different potential competitors (Stein, 2005). On the other hand, the stimulation or repression of biosynthesis of antimicrobial metabolites helps Bacillus respond appropriately to the environment such as changes in minerals and carbon levels (Mannanov and Sattarova, 2001).

Antifungal antibiotic production is involved in disease suppression. Bacillus spp. produce many antibiotics which directly suppress plant fungal pathogens, such as Rhizoctonia solani, Botrytis cinerea, Verticillium dahliae, and Sclerotinia sclerotiorum (Raaijmakers et al., 2002; Ongena and Jacques, 2008). Some commercial Bacillus strains such as FZB42, GB03, QST713, and MBI600 produce cyclic lipopeptides (CLPs) including fengycin, surfactin, or iturin. These CLPs have been associated with suppression of plant pathogens (Joshi and Gardener, 2006). For example, iturins and surfactin were active against R. solani, and pure fengycin inhibited Pyricularia oryzae (Asaka and Shoda, 1996; Joshi and Gardener, 2006). Studies on surfactin lipopeptides showed that surfactins caused hyphal swelling and fungal growth inhibition (Vitullo et al., 2012). When growing on an agar surface supplemented with surfactins, F. oxysporum mycelia exhibited retarded growth accompanied by increased branching and rosette formation as well as hyphal swelling. The production of bacillomycin and fengycin by B. subtilis UMAF6614, UMAF6639 and UMAF8561 were implicated as the mechanism of control of powdery mildew on melon caused by Podosphaera fusca
Mycosubtilin-overproducing mutants of *B. subtilis* ATCC6633 were more effective than the wild-type strain in controlling *Pythium* damping-off on tomatoes (Leclere et al., 2005).

Disintegration of membranes is well known as the primary function of antibiotics against bacteria, fungi and oomycetes. Subtilosin A produced by several *B. subtilis* strains (Stein et al., 2002) has a macrocyclic structure with three inter-residual linkages (Marx et al., 2001), which involve permeabilized cell membrane by insertion of the peptide after interaction with a cell-surface receptor (Moll et al., 1999). The damage results in irreversible modifications leading to cell death (Aly et al., 2013). For oomycetes, zoospore germination and sporangial formation represent the important steps in the asexual life cycle and the spreading of disease, and as a result, suppressing zoospore and sporangial germination may be an efficient strategy for the management of diseases caused by oomycetes (Yoshida et al., 2001). A close correlation between the production of antifungal compounds and the prevention of zoospore germination has been reported in biocontrol of *Pythium* by *B. cereus* (Shang et al., 1999).

To date, only a small proportion of the antimicrobial compounds produced by soil bacteria have been studied (El-Hassan and Gowen, 2006). One challenge is that only 1-5% of soil bacteria can be cultured. Moreover, biosynthesis of antibiotics by microorganisms is often regulated by nutrition resources (carbon level) and greatly influenced by environmental factors (pH and temperature) in the soil.

In the context of biocontrol, a successful exploratory attempt to repress Phytophthora blight by using antagonistic *B. subtilis* has been reported on pepper (Lim
The mechanisms involving *Bacillus* spp. against Phytophthora blight on squash have not yet been adequately explored. The *Bacillus* PGPR strain IN973b significantly suppressed Phytophthora blight on squash in the greenhouse and displayed *in vitro* inhibitory activities against *P. capsici*.

The specific objectives of this study were to investigate the mechanisms involved in biocontrol of *P. capsici* on squash with *B. subtilis* strain IN937b. To evaluate the inhibitory effect of antifungal compounds from the cell-free culture of IN937b, *P. capsici* zoospore germination, hyphal growth, and its effect in detached leaf assays were determined. To evaluate the role of antifungal compounds secreted by *B. subtilis* IN937b in the inhibition of *P. capsici*, we set out to determine the gene that is responsible for biosynthesis of Subtilosin A like lipitibiotics and its structural characteristics.

**Materials and methods**

**Dual Culture Assay**

All the bacterial isolates were screened for antagonism against *P. capsici* on potato dextrose agar (PDA) plates using the dual culture technique (Yoshida et al., 2001). One plug of *P. capsici* isolate #151 mycelium (6-mm diameter) was placed in the center of PDA medium. The bacterial strain was streaked on the other side of medium 3 cm away from the center. The plates were incubated at 25 °C for 7 days after which the inhibition zone was measured and recorded.

**Antibiotic Extraction and Analysis**

For isolation of the antagonistic compounds responsible for inhibiting fungal growth, strain IN937b was cultured in No.3S medium (Tsuge et al., 2005) and incubated at 28°C on a rotary shaker incubator at 150 rpm for 2 days. The culture was centrifuged
at 3,000 rpm for 30 min and the supernatant was collected and acid precipitated as
described in Chapter 3. The methanol (HPLC grade) extracts were further analyzed by
HPLC for preliminary characterization.

**Antifungal Activity of Methanol Extracts Against Phytophthora capsici**

**Mycelial Growth.** Crude extract of IN937b cell-free culture was dried in a clean
fume hood (Labconco Corporation, Kansas City, Mo). The dry matter was dissolved in
the methanol (40 mg/mL). The effective concentration (EC$_{50}$) of the extracts was
determined for *P. capsici* by serial dilution. The EC$_{50}$ was considered to be the lowest
concentration of the methanol extract that caused 50% inhibition of fungal growth. The
corn meal (CM) agar was amended with methanol extracts at a final concentration of 0,
0.2, 0.4, 0.8, 1.0 mg/mL. An agar plug (6 mm in diameter) was taken from the colony of
*P. capsici* on PARPH medium and placed at the center of CM agar amended with
methanol extracts. Each treatment had 3 replicates. All the plates were incubated at 25
°C for 9 days. The diameter of the colony was measured every 3 days after incubation.
The inhibition rate was defined as $\text{Inhibition rate(\%)} = \frac{[N_0 - N_1]}{N_0} \times 100\%$,
where $N_0$ is the mycelium diameter at a final concentration of 0 mg/mL, and $N_1$
represents the mycelial diameter of colonies on the medium amended with methanol
extracts at different concentrations.

**Zoospore Germination.** Zoospore suspensions were prepared using the
method described in Chapter 2. One hundred microliters of zoospore suspension were
mixed with 100 µL of methanol extracts at the final concentration of 0, 10, 20, 30, or
40 mg/mL (total volume is 200 µL) for the extracts. The mixture was flooded on the CM
agar and placed in the hood to allow to air dry. The plates were incubated at 25 °C for 3
h and the percentage of zoospore germination on each plate was determined by
viewing under a microscope at 400× magnification. The relative proportion of germinated zoospores was calculated (Keinath, 2007) by dividing the number of germinated zoospores by the total number (50) of zoospores counted on each plate. Two plates were used for each concentration as replicates and the experiment was conducted twice under the same conditions.

**Sporangial Formation.** *Phytophthora capsici* was grown on plugs in the DI water with the methanol extracts added at final concentrations of 0 and 40 mg/mL of crude extract. Four Petri dishes were used for each concentration and were placed under the light (one 20-watt General Electric F20T12-CW linear fluorescent lamp) at room temperature to induce sporangial formation. After 48 h, the plug was examined for sporangial formation under a dissecting microscope at 70× magnification. The number of sporangia formed on the plug was counted.

**Detached Leaf Assays**

Detached leaf assays described by Chiou and Wu (2001) were modified to investigate the direct and indirect effects of methanol extracts on *P. capsici* on the leaves. Young, fully developed leaves were collected from 2-week-old squash plants and the leaf surfaces were rinsed three times with sterile DI water. Twenty microliters of methanol extracts were dropped onto the detached leaves, and leaves were placed in the Petri dishes supplemented with moist filter paper. Twenty microliters of zoospore suspensions (10⁴ zoospore/mL) were placed on leaves 3 cm away from the site where methanol extracts were applied. The leaves were placed under the light at room temperature for 7 days. Disease severity was visually assessed using a scale of 0-5, where 0 = no symptoms, 1 = less than 10%, 2 = 10-25%, 3 = 50%, 4 = 51-75%, and 5 =
75-100% of leaves covered with brown lesions (Chiou and Wu, 2001). Leaves treated with methanol served as a non-treated control. Each treatment had three replications.

**HPLC/MS Assays of Surfactin Production**

Subtilosin A was quantified using HPLC/ESI-MS by Dr. Jodie V. Johnson as described in Chapter 3.

**DNA Extraction and PCR Analysis**

*Bacillus* PGPR strain IN937b was inoculated into 10 mL of LB medium and incubated on a rotary shaker incubator at 150 rpm overnight at 28 °C. DNA extraction was carried out using the Qiagen QIAamp DNA mini kit according to the manufacturer’s instructions. PCR reactions were carried out based on the methodology and specific primers established by Velho et al. (2013). The following primers were used for amplifying the subtilosin A (*sboA*) gene that are critical for production of the antimicrobial peptides subtilosin A:

\[
\begin{align*}
sboA-f: & \quad 5'-\text{CATCCTCGATCACAGACTTCACATG}-3' \\
sboA-r: & \quad 5'-\text{CGCGCAAGTAGTCGATTTCTAACAC}-3'.
\end{align*}
\]

The PCR reaction was performed in a final volume of 25 µL containing 2.5 µL of 10X PCR buffer, 0.5 µL of dNTP mix, 1.0 µL each of PCR primers, 0.5 µL of Taq DNA polymerases, and 1 µL each of template genomic DNA. Amplification was done with a conventional thermal cycler (BioRad, Laboratories, Hercules, CA) with an initial denaturing at 94 °C for 1 min, annealing at 50 °C for 30s and extension at 72 °C for 1 min, for a total of 35 cycles, and followed by a final extension of 72 °C for 10 min. Following amplification, the PCR reaction mix was separated through 1% agarose gel electrophoresis.
Results

Dual Culture Assays

To evaluate the antifungal activities of all selected *Bacillus* PGPR, bacterial strains were tested using a dual culture assay. Inhibition zones were clearly observed between the mycelial growth and bacterial colonies (Figure 4-1). Results of the dual culture assays for these strains are given in Table 4-1. Strain 1PN-19 exhibited the greatest inhibition to growth of *P. capsici* with an inhibition zone of 11 mm. *Bacillus* strains Companion, GB03, IN937a and IN937b showed moderately inhibitory activities with an inhibition zone less than 10 mm, whereas strains SE34, SE49, SE52, SE76, INR7, 1PC-11 and Actinovate AG had no effects on the growth of *P. capsici*.

Methanol Extracts of IN937b Culture

The active substances were collected from the cell-free culture of IN937b (surfactin production No. 3s medium) described in Chapter 3. At pH 2.0, the substances of antibiotic activity were precipitated from the cell-free supernatant and stabilized in acidic solution at 4 °C. The active substances were then extracted by methanol under neutral conditions (adjusted to pH=7.0). After evaporation, the dry weight of the active substances was approximately 40 mg from 1 liter of liquid medium culture.

Chromatographic Separation by HPLC

The extracts contained numerous compounds. A compound with MW 3400 Da in the extracts was abundant. At a retention time of 45-49 min, an m/z ratio for an ion had been selected for fragmentation. The compound produced m/z 1699 [M-2H]^2+ and m/z 1710 [(M-H+Na)-2H]^2+ ions in (-)ESI-MS and m/z 1701 [M+2H]^2+ and m/z 1712 [(M-H+Na)+2H]^2+ ions in (+)ESI-MS. The molecular mass of the corresponding metabolite
has the same molecular weight as subtilosin A which has been found to be produced by a great variety of \textit{B. subtilis} strains (Stein, 2005).

**PCR Detection of \textit{sbo} Genes in \textit{Bacillus} PGPR IN937b**

A 666-bp PCR product was amplified from IN937b using the primers \textit{sboA-f} and \textit{sboA-r} specific to fragment corresponding to the \textit{B. subtilis} subtilosin gene. DNA sequences analyzed by BLAST software revealed that this PCR fragment in IN937b had 100% similarity to \textit{B. subtilis} 168 (Accession No. AL009126) whose genome has been fully sequenced. The results indicated that IN937b is positive for the presence of the gene \textit{sbo} for cyclic peptide antibiotic subtilosin A synthesis. Amplification of the PCR products indicates that IN937b may produce subtilosin A. The result is consistent with the observation in the samples tested by HPLC.

**Assays of Hyphal Growth Inhibition**

The antifungal activity of the methanol extracts was confirmed from the culture of IN937b. One hundred microliters of methanol extracts (40 mg/mL) were pipetted into holes (6 mm diameter) 2 cm away from the edge of the CM agar. The methanol extracts of IN937b strongly inhibited mycelial growth of \textit{P. capsici} on CM agar 7 days after being added into the hole (Figure 4-2 A.). The average growth of the colony toward the hole was less than 5 mm, representing an inhibition of 75% compared to the unaffected colonies. When the concentration of the methanol extract solution was reduced to 10 mg/mL, the hyphae of \textit{P. capsici} were able to continue growing toward the inhibition zone, but the layer of hyphae were thinner and more branches were observed compared to the unaffected hyphae (Figure 4-2 B.).
Effect of Methanol Extracts on Mycelium Morphology

The hyphal morphology affected by the methanol extracts was examined in detail by microscopy. The tips of *P. capsici* hyphae grown on CM agar plants were normal (Figure 4-3 A.) from the edge of the mycelia, whereas the extract challenged hyphae of *P. capsici* were typically induced branching, and the hyphal tips became swollen as shown in Fig 4-3.

The EC$_{50}$ of the Extracts

The 0.2, and 0.4 mg/mL methanol extracts had no effects on mycelial growth of *P. capsici* (Table 4-1). None of the extracts completely inhibited mycelial growth. The extracts at 0.8 and 1.0 mg/mL had a stronger effect on the mycelial growth than the other concentrations. The estimated EC$_{50}$ of the extracts was 1.0 mg/mL.

Effect of Methanol Extracts on Zoospore Germination

Zoospores began to germinate 2 h after incubation at 28 °C. As the concentration of methanol extracts increased, a prominent reduction in the percentage of germinated zoospores was observed (Figure 4-4). The greatest inhibition (46.2%) was achieved with the 40 mg/mL extract, and none of the tested concentrations completely inhibited *P. capsici*.

Effect of Methanol Extracts on Sporangial Formation

For the assessment of the inhibitory effects of methanol extracts on sporangial formation, *P. capsici* hyphae were induced under the light for sporangial formation for 2 days at room temperature. Normal sporangia with an intact shape were present in DI water. The hyphae treated with methanol extracts had deformed and swollen tips, the same as the observations for mycelial growth inhibition (Figure 4-5). The 40 mg/mL
methanol extract completely inhibited sporangia formation (Table 4-1), and adversely affected the production of sporangia.

**Effect of Methanol Extracts on Detached Leaf Infection**

The methanol extracts were evaluated for their potential to suppress Phytophthora blight using a detached leaf bioassay (Figure 4-6). Fully expanded young leaves of squash plants from the greenhouse were used in this experiment. The 40 mg/mL extract significantly ($p \leq 0.05$) inhibited the development of Phytophthora blight compared to the methanol control (Table 4-3). No disease developed on leaves treated with the 40 mg/mL extract.

**Discussion**

*Bacillus subtilis* strain IN937b isolated from tomato rhizosphere (Murphy et al., 2000) showed inhibition activities against *P. capsici* on PDA plates (Zhang et al., 2010). Subtilosin A, an antimicrobial peptide produced by *B. subtilis* IN937b was found to be involved in reduction in zoospore germination, sporangial formation and hyphal growth of *P. capsici*, and *in vitro* the suppression of lesion expansion on the detached leaves after being challenged with *P. capsici*.

*B. subtilis* is well known for producing various antimicrobial substances as secondary metabolites to inhibit plant pathogens (Mannanov and Sattarova, 2001). Antibiotics secreted by *B. subtilis* include non-ribosomally synthesized peptide antibiotics (e.g. surfactin and mycobacillin) and ribosomally synthesized precursor lantibiotics (e.g. subtilin and subtilosin A) (Stein et al., 2002; Bais et al., 2004b; Leclere et al., 2005; Arguelles-Arias et al., 2009).

The antifungal activity of bacteria might be due to competition for nutrients with other microorganisms (Janisiewicz et al., 2000). *Bacillus* strains which contribute to
biologically controlling plant diseases synthesize antibiotic compounds that suppress or kill other microbes, modify microbial attachment to plant tissue surfaces, and improve survival of the *Bacillus* cells in their habitat. The findings in our studies, together with the fact that biocontrol efficiency of *Bacillus* strains were closely correlated with antibiotics production, strongly support the relevance of antibiosis as an important factor involved in the protection of squash against *P. capsici* by these PGPR strains.

**Hyphal Growth Inhibition**

Sixteen *Bacillus* strains were tested for their ability to inhibit hyphal growth of *P. capsici* on PDA plates. These strains varied in their capacities against *P. capsici*. Strain IN937b showed moderate levels of fungal inhibition (inhibition zone was approximately 5 mm). Thus, the antibiotics produced by IN937b were presumably partially responsible for suppressing the hyphal growth of *P. capsici* (Ahmed et al., 2003; Zhang et al., 2010).

Methanol extract of strain IN937b was increased inhibition of hyphal growth compared to IN937b cells did on PDA plates. In the present study, antibiotics produced by IN 937b were condensed by precipitation with HCl from the culture supernatants and were extracted from the pellet with methanol (Vater et al., 2002). The condensed extracts had a 40-fold increase in inhibition of hyphal growth compared with the culture of IN937b. The very weak antifungal activity displayed by the culture of IN937b suggested that antibiotics are produced in very small quantities. The methanol extracts could have resulted in the enrichment of antibiotics and therefore led to the increased antifungal activity. Application of antibiotics rather than bacterial cells into agricultural systems may improve disease control (McManus et al., 2002).
Zoospore Germination and Sporangial Formation

We demonstrated that zoospore germination and sporangial formation of \textit{P. capsici} were significantly inhibited by the methanol extracts of IN937b. Zoospore germination is believed to be the most important phase in the asexual life cycle and spread of \textit{P. capsici} (Judelson and Blanco, 2005). In the present study, the extracts were able to reduce \textit{P. capsici} zoospore germination with results similar to those previously reported (Kone et al., 2009). Microscopic observations revealed that sporangial formation of \textit{P. capsici} was completely inhibited in DI water pretreated with the extracts. These findings support the hypothesis that secretion of antifungal compounds is the primary mechanism contributing to the suppression of \textit{B. subtilis} strain IN937b against \textit{P. capsici}. Antibiotics are capable of modifying the fungal membrane permeability causing enlargement of the mycelium and distortion of sporangia and zoospores (Leelasuphakul et al., 2008).

Detached Leaf Assays

In this study, we used detached leaves to assess disease severity on squash leaves treated with the extracts produced by IN937b. Although conditions in this assay were highly favorable to \textit{P. capsici} infection, the extracts of IN937b, applied before pathogen challenge, completely suppressed the disease development on the attached leaves. However, if treated with the extracts simultaneously or after the pathogen inoculation, the extracts failed to suppress the disease development. Therefore, antibiosis by the strain IN937b plays a major role in the suppression of Phytophthora blight in squash. Inhibition of \textit{P. capsici} on squash leaves by the extracts observed in this study suggests that the extracts do not have a therapeutic effect but exert preventive activities against the Phytophthora blight disease (Curtis et al., 2004). Once
the pathogen is established in the plant tissues, the effect of suppression on mycelial growth diminished.

**Identification of Subtilosin A**

Subtilosin A was isolated from IN937b culture, and identified by HPLC/ESI-MS. Subtilosin A, with a molecular mass of 3399.7 Da (Marx et al., 2001), is a cyclic polypeptide that is ribosomally synthesized in *Bacillus* spp.. HPLC analysis showed that there were large quantities of subtilosin A in the methanol extracts. The initial pH and culture temperature are critical factors for microbial growth and metabolic biosynthesis, and they substantially affect antibiotic production (Gong et al., 2006).

Subtilosin A targets cell membranes to form transient pores inducing lipid-perturbation and contributing to its antibiotic activity. It has been reported that subtilosin A involves permeabilization of target cell membranes (Thennarasu et al., 2005). Subtilosin A promotes internal osmotic imbalance and wide cytoplasmic disorganization through abundant vacuoles formation. The aggregation of cytoplasm increases along with a loss of characteristic organelles. These changes result in the plasmolysis of fungal cells and ultimately cause morphological damage visible in sporangia and zoospore germination.

The exploitation of antibiotics as active ingredients of pesticides is promising in developing novel fungicides to control crop diseases. Development of compounds suitable for agricultural use as pesticide ingredients requires intrinsic toxicity and stability. Therefore, exploring less toxic and more stable compounds may be one of the most promising strategies for developing novel biocontrol agents. *B. subtilis* strains could be important candidates for biocontrol agents because they are known to produce more than two dozen antibiotics (Stein, 2005).
In conclusion, we have demonstrated that secondary metabolites secreted by *B. subtilis* IN937b strongly inhibited the growth of *P. capsici*. Although antibiosis seems to be an important mechanism involved in biocontrol activity of the active compounds. Further studies of the modes of action of these secondary metabolites are currently underway in order to improve their efficacy.
Table 4-1. Antagonism of *Bacillus* PGPR strains against *P. capsici* on PDA plates

<table>
<thead>
<tr>
<th>Bacillus strains</th>
<th>Isolates of <em>P. capsici</em></th>
<th>#121</th>
<th>#146</th>
<th>#151</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE34</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>SE49</td>
<td>-</td>
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<tr>
<td>SE52</td>
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<td>SE56</td>
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<tr>
<td>SE76</td>
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<tr>
<td>INR7</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>1PC-11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Actinovate AG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1PN-19</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>BUEX 1216S</td>
<td>++</td>
<td>++</td>
<td>++</td>
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</tr>
<tr>
<td>BUEX 1216C</td>
<td>++</td>
<td>++</td>
<td>++</td>
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</tr>
<tr>
<td>Companion</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>GB03</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>IN937b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>IN937a</td>
<td>++</td>
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</tr>
</tbody>
</table>

- Represents no inhibition zone. + Represents 1–5 mm wide inhibition zone, ++ represents 6–10 mm wide zone, +++ represents more than 10 mm wide zone. Values are average of three replications.
Table 4-2. Effect of Methanol Extracts of IN937b on hyphal growth of *P. capsici*

<table>
<thead>
<tr>
<th>Concentration (mg/mL)</th>
<th>Diameter of mycelial colony (cm) and inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3 (%)</td>
</tr>
<tr>
<td>0.0</td>
<td>3.9 a</td>
</tr>
<tr>
<td>0.2</td>
<td>3.7 b</td>
</tr>
<tr>
<td>0.4</td>
<td>2.9 c</td>
</tr>
<tr>
<td>0.8</td>
<td>2.7 d</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0 e</td>
</tr>
</tbody>
</table>

*a* All the plates were incubated at 25 °C for 9 days. The diameter of mycelial colony was measured 3, 6 and 9 days after incubation.

*b* Each value represents the mean of three replicates. Means followed by the same letter within each column were not significantly different according to Fisher’s protected LSD test (p = 0.05).
Table 4-3. Effect of Methanol Extracts of IN937b in Detached Leaf assays

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease severity $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>Methanol (CK)</td>
<td>1.1±0.3 $^b$ a</td>
</tr>
<tr>
<td>Methanol extracts (40 mg/mL)</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$Disease severity was evaluated based on the scale of 0-5: where 0 = no symptoms; 1 = less than 10%; 2 = 10-25%; 3 = 50%; 4 = 51-75%; 5 = 75-100% of leaves covered with lesions.

$^b$Each value represents the mean of four replicates. Means followed by the same letter within each column were not significantly different according to Fisher’s protected LSD test (p = 0.05).
Figure 4-1. Antagonism of *Bacillus* spp. against *P. capsici* on PDA plates. *In vitro* assays of *Bacillus* strains to suppress *P. capsici* were conducted on PDA plates. A 6 mm-diameter mycelial plug of *P. capsici* was placed in the center of PDA. The bacterial strain was streaked a 4 cm long line 3 cm away from the center of plug. The plates were incubated at 25 °C for 7 days. A) strains IN937b and IN937a, CK: only mycelial plug of *P. capsici* on the PDA, B) strains SE76 and IPC-11, CK: only mycelial plug of *P. capsici* on the PDA.
Figure 4-2. Antagonism of methanol extracts of IN937b against \textit{P. capsici} on CM agar plates. One hundred microliters of methanol extracts were pipetted into each of the holes (6 mm diameter) 2 cm away from the edge of the CM agar. A 6 mm-diameter mycelial plug of \textit{P. capsici} was placed in the center of CM agar plates. A) 100 \( \mu \text{L} \) methanol extracts (40 mg/mL), B) 100 \( \mu \text{L} \) methanol extracts (10 mg/mL). A thin layer and more branches were observed close to the hole (black arrow).
Figure 4-3. Effect of methanol extracts of IN937b on morphology of *P. capsici*. Effect of methanol extracts on morphology of *P. capsici* under a light microscope. A) normal mycelia of *P. capsici* observed at the edge of the plate away from the extracts, B) abnormal mycelial swollen and branching of *P. capsici* towards to the extracts. Distorted morphology in the presence of methanol extracts (white arrows).
Figure 4-4. Effect of methanol extract concentrations of IN937b on zoospore germination. One hundred microliters of zoospore suspensions (10^5 zoospores/mL) mixed with 100 µL methanol extracts at the final concentration of 0, 10, 20, 30, or 40mg/mL. Data are the percentage of germinated zoospores after 3 h incubation at 25 °C. The equation of regression analysis represents a dose response of germinated zoospores to the extract concentration, where x is the extract concentration and y is the zoospore germination rate. Each value represents the mean of four replicates. Means followed by the same letter within each column were not significantly different according to Fisher’s protected LSD test (p = 0.05).
Figure 4-5. Effect of methanol extracts of IN937b on sporangial formation. The mycelial plug was placed in 15 mL DI water with 100 µL methanol extracts (40 mg/mL). The plates were placed under the light at room temperature to induce sporangial formation. After 48 h, the number of sporangia was examined under a dissecting microscope. A) Sporangia were observed without methanol extracts, B) No sporangia were found and swollen tips of hypha existed in treatment with methanol extracts.
Figure 4-6. Effect of methanol extracts on detached leaf infection. Squash leaves (less than 9 cm) were collected from 2 week-old plants. Twenty microliters of methanol extracts were dropped on to the detached leaves and placed in the Petri dishes supplemented with moist filter paper. One day later, 20 µL zoospore suspensions (10^4 zoospore/mL) were dropped on to the leaves 3 cm away from the site that treat with methanol extracts. A) Rating scale of disease severity. Disease severity was evaluated using a scale of 0-5: where 0 = no symptoms; 1 = less than 10%; 2 = 10-25%; 3 = 50%; 4 = 51-75%; 5 = 75-100% of leaves covered with brown lesions, B) Symptom development recorded 5 days after pathogen inoculation, C) Symptom development recorded 7 days after inoculation, D) Symptom development recorded 9 days after inoculation.
CHAPTER 5
ELICITATION OF PLANT SYSTEMIC DEFENSE BY *Bacillus* PGPR STRAINS

Introduction

Plants are constantly exposed to various pathogens. In order to prevent potential plant infection by pathogens, plants possess an immune system that recognizes the pathogens and activate defenses that protect plants from infectious fungi, oomycetes, bacteria, and nematodes, etc. (Kuc, 1982). A first line of defense is called pathogen-associated molecular pattern (PAMP) - triggered immunity (PTI), resulting in non-host resistance that prevents further pathogen invade (Jones and Dangl, 2006). PTI leads to fast defense responses via synthesis of toxins, antifungal proteins, and chemical signals during initial stages of pathogen infection (Nicaise et al., 2009). However, some pathogens develop strategies to suppress PTI. Therefore, plants have to develop a second line of defense called effector-triggered immunity (ETI), which relies on systemic signals produced at and dispersed from infection sites (Amil-Ruiz et al., 2011). In general, ETI is associated with a hypersensitive response (HR) and a systemic activation of plant defenses at the site of signal perception with pathogenesis-related (PR) proteins, phytoalexins and reactive oxygen species.

Plant defenses are not only activated in response to pathogen infection, but also can be stimulated by beneficial microbes. After exposure to biotic stimuli provided by PGPR, plants can acquire enhanced levels of resistance to subsequent pathogen attack. This phenomenon is commonly referred to as induced systemic resistance (ISR) (Kloepper et al., 2004). Biological control using introduced PGPR with the capacity to elicit ISR against plant pathogens has been extensively studied over the past two decades in greenhouse and field trials (Yan et al., 2002). In many cases, ISR elicits
plant immunity against multiple plant pathogens (Kloepper et al., 2004). For example, select mixtures of compatible Bacillus spp. with the capacity to elicit ISR protect the hosts against infection by pathogens Ralstonia solanacearum on tomato, Colletotrichum gloeosporioides on pepper, and Rhizoctonia solani on green kuang futsoi (Brassica chinensis var. parachinensis) (Jetiyanon and Kloepper, 2002).

A common feature of resistance responses induced by PGPR is “priming”, a state in which plants treated with PGPR display fast and strong activation of defense responses upon pathogen challenge. A priming effect is when PGPR strains cause the plant to be in an “alert” state to detect pathogens with the response occurring faster and/or stronger compared to plants not previously exposed to the priming stimulus (Yang et al., 2009). Primed plants display a unique physiological status that can respond rapidly to pathogen attacks (Conrath et al., 2006). For example, Bacillus sp. CHEP5 and Pseudomonas sp BREN6 strains reduced root and stem wilt disease severity caused by Sclerotium rolfsii on peanut (Arachis hypogaea L) (Laura Tonelli et al., 2011). PGPR-mediated ISR caused a faster increase in phenylalanine ammonia-lyase and peroxidase levels in peanut plants compared to non-treated control plants after pathogen challenge, indicating that a state of priming was activated by treatment with CHEP5 and BREN6. However, ISR usually does not confer adequate control, but provides long-lasting increased resistance against a broad range of pathogens (Akram et al., 2008).

ISR occurs when plants respond to an appropriate elicitor from the tissues surrounding the initial infection site, which spreads throughout the plant via the emission of molecular signals that reach distant tissues triggering increased resistance to the
pathogens. The signaling of ISR is controlled by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Pieterse et al., 2002). *Paenibacillus alvei* K165 primed for enhanced SA-dependent defenses (Tjamos et al., 2005), while some other Bacillus spp. such as *Bacillus cereus* AR156, were able to prime both the SA and the JA/ET pathways (Niu et al., 2011). Recently, lipopolysaccharides, 2,4-diacetylphloroglucinol, and siderophores produced by PGPR have been found to be responsible for ISR (Henry et al., 2011). All these signaling molecules can distribute from local to far sites.

To defend against pathogen attack, there are two defense pathways that are generally associated with an enhanced level of resistance conferred by ISR: the phenylpropanoid pathway and the oxylipin pathway. Phytoalexin synthesis induction through stimulation of the expression of genes involve phenylalanine ammonia-lyase (PAL). The first step of the oxylipin pathway is catalyzed by PAL, which activates and catalyzes phenylalanine to cinnamic acid, the precursor of lignin, salicyclic acid, and phytoalexin phenylpropanoids, leading to secondary metabolites such as phytoalexins that are toxic to fungi (Silva et al., 2004a). PAL is also involved in lignin biosynthesis and accumulation of phenolics in response to fungal infection. Enzyme stimulation as part of ISR is generally activated after pathogen infection (Choudhary et al., 2007).

ISR is associated with an oxidative burst (Bargabus et al., 2003) and cell wall reinforcement (van Loon, 2007) by accumulation of a defense related enzyme peroxidase (PO). PO shows affinity to cellular lignification and has direct antimicrobial activity in the presence of hydrogen peroxide (Silva et al., 2004b). The oxidative burst is the earliest event in plant defense response in both compatible and incompatible plant-pathogen interactions as well as in rhizobacteria - plant interactions (Jourdan et al.,
In general, the transient primary burst is nonspecific and has no effect on disease progression. Only during incompatible interactions, more prolonged peaks of hydrogen peroxide production lead to specific recognition (Van Breusegem et al., 2001). The enforcement of cell walls by lignification involves blocking of fungal pathogen penetration. Upon challenge infection with the fungus, the root cell walls are rapidly strengthened at the sites of attempted fungal penetration through appositions that contain callose as well as phenolic materials and fungal ingress is effectively prevented (Yoshida et al., 2005). Tobacco plants treated with \textit{B. pumilus} SE34 exhibited newly formed barriers beyond the site of fungal infection. These barriers were cell wall appositions that contained large amounts of callose and were infiltrated with phenolic compounds (Zhang et al., 2002). Phenolic compounds accumulated in host cell walls, intercellular spaces, and on the surface and inside of the invading pathogen’s hyphae (Nakkeeran et al., 2006).

ISR induced by \textit{Bacillus} PGPR was shown to be generated in whole squash plants and a split-root system. In studies of protection of cucumber against diseases caused by soil-borne fungi, a split-root assay was developed in which the inducing bacteria and the pathogen were simultaneously inoculated on separate halves of roots, and then planted in separate pots (Liu et al., 1995). ISR was expressed as a delay in symptom development, reduced disease severity, and reduced disease incidence compared to the non-bacterized controls. In a previous study, the compatibility between \textit{P. capsici} and susceptible pepper plants (\textit{Capsicum chinense} Jacq) could be a non-host compatible interaction due to a consistent increase activity of defense enzymes a few hours after inoculation with \textit{P. capsici} (Goretty Caamal-Chan et al., 2011). It appears
that *P. capsici* has the ability to block the defense response and to manipulate the metabolism of the host by its effectors for successful infection (van Loon et al., 2006).

The objectives of this study were: (1) to understand the nature of ISR by *Bacillus* PGPR IN937b and SE76 in squash plants with split-root assays by measuring the levels of PAL and PO, (2) to evaluate the potential host defense response in squash plants against *P. capsici* by monitoring PAL and PO activities, and (3) to analyze the changes in enzyme activity of PAL and PO by treatment with *Bacillus* PGPR applied at different squash growth stages.

**Materials and Methods**

**Inoculum Preparation and Inoculation**

Inoculum of *P. capsici* isolate #151 was prepared according to Chapter 2. The concentration of zoospores was determined under a microscope using a hemocytometer.

For foliar inoculation, the concentration of *P. capsici* inoculum was adjusted to 10^3 zoospores/mL using DI water. Plant leaves were sprayed with 15 mL of the inoculum until run off. Each inoculated plant was covered with a plastic bag to maintain high moisture for 2 days. Inoculated plants were maintained on benches in the greenhouse with high soil moisture by hand watering daily.

**Application of Bacillus PGPR Treatment in the Greenhouse**

PGPR strains IN937a, IN937b and SE76 were evaluated for their ability to systemically induce resistance against *P. capsici* in the greenhouse. Two-week-old squash plants were treated with 20 mL of PGPR suspensions prepared according to Chapter 2 at 1×10^8 cfu/mL as a soil drench. One week later, the same treatment with PGPR were applied to the seedlings for a second time.
**Split-Root Assay**

To set up the split-root systems, germinated seeds were planted in 60-well plastic trays filled with potting mix. After 10 days, the roots were washed with running tap water. The roots were then split evenly with a scalpel blade and each half of the root system of each seedling was quickly moved into two adjacent plastic pots, each containing potting mix. Each pot was separated to guarantee physical separation of each half root. The plants were placed on the bench in a greenhouse.

One week after roots were split, one half of the root system of each plant was divided into groups, each treated with a different inoculum. Group 1: one half of the root system of each plant was treated with 20 mL of IN937b suspensions at $1 \times 10^8$ cfu/mL applied as a soil drench labeled as IN937b-2. The other half of the root system of each plant were treated with same amount of DI water labeled as IN937b-1. Group 2: One half of the root system of each plant was treated with 20 mL of ASM at 30 mg/L applied as a soil drench labeled as ASM-2. The other half of the root system of each plant were treated with same amount of DI water labeled as ASM-1. Group 3: Both halves of the root system of each plant were treated with DI water labeled as non-treated control (CK-1 and CK-2).

**Enzyme Activity Assays**

Samples of fresh squash leaves and roots were taken from each of the pots 1, 3, 5, 7, and 10 days after IN937b treatment. The samples were washed with running tap water, dried and 0.5 g of each was ground in liquid nitrogen in a mortar with a pestle. The ground tissue was then mixed with 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant used for the enzymatic activity assay was stored in a 1.5 mL Eppendorf tube at -20 °C. Healthy plants with no
PGPR or pathogen challenge were used as a background. Background PAL and PO activity was found to be same during experimental period.

**Enzyme Activity Assays**

**Peroxidase (PO)** Fifty microliters of roots or leave extracts were added to 2.95 mL of PO reaction buffer consisting of 50 mM sodium phosphate buffer (pH 6.0), 60 mM guaiacol and 0.6 M H₂O₂ (Chen et al., 2000). The reaction was measured for 90 s (recording the value every 15 s) at wavelength of 470 nm with a spectrophotometer (UNICO SpectroQuest TM 4802 UV/VIS Double Beam, United Products & Instruments Inc., Dayton, NJ). One unit of enzyme activity was defined by a change in absorbance of 0.01 for 1 g fresh weight per minute.

**Phenylalanine ammonia-lyase (PAL)** One hundred µL of crude plant tissue (roots or leave) extracts were mixed with 1.9 mL of 0.015 M L-phenylalanine and 0.1 M Tris-HCl buffer solution (pH 8.5) (Chen et al., 2000). The mixture was placed in a water bath at 40 °C for 30 min. PAL activity was measured spectrophotometrically at a wavelength of 290 nm. One unit represents the conversion of 1 µmol L-phenylalanine to cinnamic acid for 1 g fresh weight per minute at 40 °C.

**Results**

**Induction of Systemic Resistance by PGPR with *P. capsici* Challenge**

Soil treatment with IN937b resulted in a significant (*P*≤0.05) visible reduction in lesion development (both number and size) compared with the non-treated control 7 days after inoculation (Table 5-1). SE76 failed to elicit ISR because the number and size of lesions increased as much as in the non-treated control. ASM was the most effective treatment in this experiment to induce ISR, as it completely stopped the occurrence and spread of symptoms.
Treatment with IN937b at $10^8$ and $10^7$ CFU/mL significantly ($P \leq 0.05$) reduced the size of lesions compared to the non-treated control 7 days after pathogen inoculation (Table 5-2). The greatest reduction in lesion size by IN937b was at $10^8$ CFU/mL, where there was more than 70% reduction compared to the non-treated control plants. IN937b at $10^7$ and $10^6$ CFU/mL had no effect on disease development compared to the non-treated control. ASM completely inhibited the disease during the period of this experiment.

**Increased Activities of PO and PAL Induced Locally by PGPR Treatment with *P. capsici* Challenge**

Compared to the non-treated plants, SE76 induced significantly greater levels of PAL and PO activities in plants roots 3 days after PGPR treatment (Figure 5-1 and Figure 5-2). When squash roots were challenged with *P. capsici*, significantly greater PAL activities were observed in all PGPR treatments and ASM 3 DAI compared to the non-treated control (Figure 5-3). PAL activities were significantly greater in treatments of ASM and IN937b than the non-treated control 5 DAI. In general, levels of PAL increased drastically 1 DAI in all treatments due to the rapid response of the squash plants to the pathogen. However, PAL activities 3 DAI significantly decreased in all treatments except for the background control compared to those at 1 DAI.

*Bacillus* PGPR IN937b and SE76 induced significantly greater levels of PO activities 5 DAI compared to the non-treated control (Figure 5-4). PO activities reached the highest levels 3 DAI in all treatments and then decreased 5 DAI. In the non-treated control plants, PO activities significantly increased 1 and 3 DAI when the pathogen started to invade squash plants, but dramatically declined 5 DAI when stem wilting started to occur. Treatment with IN937a had no effect on PO activities, while ASM
treatment resulted in significantly greater levels of PO activities 3 and 5 DAI compared to the non-treated control plants.

**Systemically Induced Enzyme Activity as Determined by Split-Root System without *P. capsici* Challenge**

Data from the split-root system assays showed that IN937b not only locally increased levels of PO and PAL activities in the root half that was bacterized, but also systematically increased PO and PAL activities in the other half (distant side) of the root system which was only treated with water at 10 days after IN937b treatment (Figure 5-5 and 5-6).

**Increased PAL and PO Activities Systemically in Response to *P. capsici* in Different Seedling Stages**

Treatment with IN937b and SE76 induced significantly higher PAL activity in plant leaves compared to the non-treated control 5 DAI for young and older plants (Fig 5-7). When first PGPR treatment was applied 17 days after emergence, significantly greater PAL activity were detected 5 DAI in all PGPR treatments and ASM compared to the non-treated control. Similarly, significantly greater PAL activity were detected 5 DAI in all PGPR treatments compared to the non-treated control when the first PGPR treatment was applied 10 days after emergence.

Squash plants treated with IN937b, SE76 and ASM induced significantly greater levels of PO activity in the leaves compared to the non-treated control 5 DAI in the young plants with the first PGPR treatment 10 days after emergence (Fig 5-8). In older plants for which the first PGPR treatment was performed 17 days after emergence, there was no difference of PO activity were observed in all treatment 5 DAI which the first PGPR treatment was applied 10 days after emergence. PO activity was greater in leaves treated with ASM for both young and older plants.
Discussion

The purpose of this study was to demonstrate that *Bacillus* PGPR strains are able to trigger ISR in squash plants against *P. capsici*, and to reveal the underlying mechanisms of PGPR suppression of Phytophthora blight disease. *Bacillus* PGPR IN937b applied in the rhizosphere reduced the number and size of lesions on the leaves infected by *P. capsici*. Since PGPR and the pathogen were spatially separated, the protection is not associated with the ability of the PGPR strains to inhibit fungal growth and, therefore, it may be, at least partially, attributed to ISR-mediated protection. The results from this study demonstrated that both *Bacillus* PGPR and the pathogen were able to affect activities of defense-related enzymes, i.e. PAL and PO.

Actigard® (ASM) is a well-known activator of host resistance in many plants (Louws et al., 2001; Kone et al., 2009). In this study, ASM induced significantly systemic resistance in squash, resulting in elimination of *P. capsici* infection. It suggests that ASM may induce resistance more effectively than *Bacillus* PGPR (Barilli et al., 2010).

**Defense-Related Enzymes**

Plants suppress pathogens by building lines of defense and develop an array of cellular mechanisms to defend themselves against invading pathogens (Ramamoorthy et al., 2001). Plants can recognize a pathogen and limit it to the site of the infection (Jones and Dangl, 2006), and stop pathogen infection and movement (Takemoto et al., 2003).

In general, increases in enzyme activities related to plant defenses are important indicators in response to disease development. PO is an oxido-reductive enzyme involved in the wall binding process (Viswanathan and Samiyappan, 2002). PO has been implicated in the last enzymatic step of lignin biosynthesis, which is the oxidation...
of hydroxyl which is subsequently coupled to lignin polymer. PO is also believed to be involved in a number of physiological processes that may contribute to resistance including cross linking of extensin monomers and lignification and they are also associated with deposition of phenolic compounds into plant cell walls during resistance reactions (Passardi et al., 2004). To be effective against a pathogen, defense mechanisms, including increased PO activity, should be rapidly elicited in response to infection. One of the immediate host responses to infection is the accumulation of phenolics at the infection site. Similar results were also obtained in a previous study, where microbial mixture of Bacillus strains IN937a and IN937b were found to induce maximum SOD and PO activities compared with the non-treated control (Jetiyanon, 2007).

PAL is the first enzyme in the phenylpropanoid biosynthesis pathway leading to synthesis of phytoalexins or phenols, which have defense functions in plants, such as reinforcement of plant cell walls, antimicrobial activity and synthesis of signaling compounds such as salicylic acid (Wen et al., 2005). In the present study, after challenge with the pathogen P. capsici, PAL activities were positively correlated the plant’s resistance against the pathogen as indicated by reduced disease severity. PAL is essential for the synthesis of all protective substances through the phenylpropanoid pathway. Generally, induction of PAL is correlated with increased resistance in the host to pathogenic infection. PAL leads to the biosynthesis of phenols, phytoalexins, and lignins with the involvement of PO.

Increased PAL and PO activities in plants by PGPR enhance the production of phenolic compounds, and hence induce development of physical and chemical barriers
to pathogen attack. Increased enzyme activities in PGPR-mediated ISR might be one of the defense mechanisms involved in suppression of *P. capsici*. Phenolics generally fungitoxic and increase the mechanical strength of the host cell walls. Lignin is a phenolic polymer which is difficult for pathogens to breach and has been implicated in plant defense against pathogens.

In the present study, induced responses in squash plants occurred in a short time period. Resistance activated by PGPR did affect pathogen infection. However, following the early and increased expression of defense enzymes, PAL activity dramatically decreased after *P. capsici* infection, suggesting that the pathogen had the ability to suppress induced defense responses in plants by interfering with signaling cascades and detoxification of phytoalexins (El-Hadrami et al., 2009).

**Locally Induced Enzyme Activity**

When the squash roots were treated with *Bacillus* PGPR strains and inoculated with *P. capsici*, the differential induction of resistance may have been activated depending on the induction site. When roots were treated with *Bacillus* PGPR, metabolic processes may have produced reactive oxidative oxygen species (ROS) such as H₂O₂, O₂ and OH (Demidchik, 2012). The production of toxic oxygen derivatives increased as a result of infection by any foreign microbes. PGPR strains are able to alleviate ROS via increased activities of antioxidant enzymes such as PO in plants, and establish a relationship with plant roots without being harmful to roots (Zamioudis and Pieterse, 2012). In the present study, the observed increased localized PO levels in squash roots by bacterization are in agreement with the reports that cucumber roots with PGPR induced localized resistance (Chen et al., 2000; Liang et al., 2011).
**Priming: Getting Ready for Pathogen Invasion**

PGPR may alter the cellular mechanisms and reprogram metabolism after they establish a relationship with plants, and induce plant resistance upon subsequent challenge with a pathogen (Van der Ent et al., 2009). Plants treated with PGPR become primed to respond faster and show stronger activation of cellular defense responses upon pathogen challenge compared with unprimed plants (Conrath et al., 2002). Treatment with *Bacillus* strains in roots also increased the activities of PO and PAL enzymes in squash leaves. This is also supported by the observations in the split-root assays that certain *Bacillus* strains induced increased PO and PAL activities in the other half root which was treated with water and distant from the treated half roots.

Older plants tended to develop disease symptoms later compared to younger plants after they were inoculated with the same amount of *P. capsici*. PAL and PO activities were greater in older plants than younger plants in the non-treated control, IN937b and ASM treatments. In general, the activities of PAL and PO increased in these treatments with reduced disease severity in squash plants.

In the present study, PAL activity was activated by *Bacillus* PGPR IN937b and SE76. It appears that the pathogen blocked the plant defense response development as previously reported (Cooper and Rao, 2006). PO and PAL activities were promptly triggered by PGPR treatments, and the levels of PO and PAL activities were faster than the chemical inducer (ASM) did, and this seems to be a consensus for PGPR treatment. PO activity is activated faster to contain the reactive oxygen species produced by host plants that respond to inhibit pathogen growth (Ishida et al., 2008).

In conclusion, higher levels of PO and PAL activities may have contributed collectively to induced resistance in squash plants against *P. capsici*, the pathogen of
Phytophthora blight, which eventually resulted in lower disease severity compared to the non-treated control.
Table 5-1. Effect of *Bacillus* PGPR on number and size of lesions in the detached leave assay under the lab conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>lesion size in cm²</th>
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<tbody>
<tr>
<td></td>
<td>Day 5</td>
<td>Day 7</td>
<td></td>
</tr>
<tr>
<td>IN937b a</td>
<td>9.0 a c</td>
<td>13.0 b</td>
<td></td>
</tr>
<tr>
<td>SE76</td>
<td>12.0 a</td>
<td>32.5 a</td>
<td></td>
</tr>
<tr>
<td>ASM</td>
<td>0.0 b</td>
<td>3.9 b</td>
<td></td>
</tr>
<tr>
<td>Non-treated control</td>
<td>11.9 a</td>
<td>44.6 a</td>
<td></td>
</tr>
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</table>

a PGPR were applied twice (1.0 × 10^8 CFU/mL; 20mL) as a soil drench. ASM (Actigard® 30 mg/L; 20 mL) was applied as a positive SAR inducer for suppressing Phytophthora blight.

b *P. capsici* inoculum was diluted to 1 × 10^3 zoospores/mL. Plants were sprayed with 20 mL of *P. capsici* inoculum until ran off. Data of the number and size of lesions were collected at 5 and 7 days after pathogen inoculation.

c Each value represents the mean of four replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05).

Table 5-2. Effect of different concentrations of IN937b on the number and size of lesions in the detached leave assay under the lab conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>lesion size in cm²</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 5</td>
<td>Day 7</td>
</tr>
<tr>
<td>937b-10^8</td>
<td>2.5 a</td>
<td>4.0 a</td>
<td>5.0 b</td>
</tr>
<tr>
<td>937b-10^7</td>
<td>3.9 a</td>
<td>5.5 a</td>
<td>8.0 b</td>
</tr>
<tr>
<td>937b-10^6</td>
<td>6.6 a</td>
<td>8.5 a</td>
<td>10.5 a</td>
</tr>
<tr>
<td>Non-treate</td>
<td>6.5 a</td>
<td>7.6 a</td>
<td>16.8 a</td>
</tr>
<tr>
<td>d control</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a PGPR were applied twice as a soil drench. The concentration of IN937b was adjusted to 10^6, 10^7, and 10^8 CFU/mL, and 20 mL was applied to each plant. ASM (Actigard® 30 mg/L; 20mL/plant) were applied as SAR control for suppressing Phytophthora blight.

b *P. capsici* inoculum was diluted to 1 × 10^3 zoospores/mL. One leaf of each plant was sprayed with 5 mL of *P. capsici* inoculum until ran off. Data of the number and size of lesions were collected at 3, 5, and 7 days after pathogen inoculation.

c Each value represents the mean of ten replicates. Means followed by the same letter within a column are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Figure 5-1. Induction of PAL by PGPR in squash roots. PAL activities in squash roots treated by PGPR only. Squash roots were sampled 1 day and 3 days after PGPR treatment. Bars represent the standard error of the mean from three replications. Means followed by the same letter are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Figure 5-2. Induction of PO by PGPR in squash roots. PO activities in squash roots treated by PGPR only. Squash roots were sampled 1 day and 3 days after PGPR treatment. Bars represent the standard error of the mean from three replications. Means followed by the same letter are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Figure 5-3. Induction of PAL by PGPR in roots against *P. capsici*. PAL activities in squash roots treated by PGPR and challenged with *P. capsici* (3 days after PGPR treatment). Squash roots were sampled 0, 1, 3 and 5 day after *P. capsici* inoculation. Bars represent the standard error of the mean from three replications. Means followed by the same letter are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Figure 5-4. Induction of PO by PGPR in roots against *P. capsici*. PO activities in squash roots treated by PGPR and challenged with *P. capsici* (3 days after PGPR treatment). Squash roots were sampled 0, 1, 3 and 5 day after *P. capsici* inoculation. Bars represent the standard error of the mean from three replications. Means followed by the same letter are not significantly different according to Fisher’s protected LSD test (*p* = 0.05).
Figure 5. Induction of PAL by IN937b in split root system. PAL activities on squash roots treated by IN937b only. CK-1: root with water treatment, CK-2: root with water treatment, IN937b-1: root with water treatment, IN937b-2: root with IN937b treatment, ASM-1: root with water treatment, ASM-2: root with ASM treatment. Squash roots were sampled 1, 3, 5 and 10 day after PGPR treatment. Bars represent the standard error of the mean from three replications. Means followed by the same letter are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Figure 5-6. Induction of PO by IN937b in split root system. PO activities in squash roots treated by IN937b only. CK-1: root with water treatment, CK-2: root with water treatment, IN937b-1: root with water treatment, IN937b-2: root with IN937b treatment, ASM-1: root with water treatment, ASM-2: root with ASM treatment. Squash roots were sampled 1, 3, 5 and 10 day after PGPR treatment. Bars represent the standard error of the mean from three replications. Means followed by the same letter are not significantly different according to Fisher's protected LSD test (p = 0.05).
Figure 5-7. Induction of PAL by PGPR against *P. capsici* in different growth stages. PAL activities in squash roots treated by PGPR and challenged with *P. capsici* (3 days after PGPR treatment). Squash roots were sampled 0, 1, 3 and 5 day after *P. capsici* inoculation. A) First PGPR treatment started at 10 days after emergence, B) First PGPR treatment started at 17 days after emergence. Bars represent the standard error of the mean from three replications. Means followed by the same letter are not significantly different according to Fisher’s protected LSD test ($p = 0.05$).
Figure 5-8. Induction of PO by PGPR against *P. capsici* in different growth stages. PO activities in squash roots treated by PGPR and challenged with *P. capsici* (3 days after PGPR treatment). Squash roots were sampled 0, 1, 3 and 5 day after *P. capsici* inoculation. A) First PGPR treatment started at 10 days after emergence, B) First PGPR treatment started at 17 days after emergence. Bars represent the standard error of the mean from three replications. Means followed by the same letter are not significantly different according to Fisher’s protected LSD test (p = 0.05).
Squash is one of widely grown and consumed vegetable crops worldwide. The large scale of squash plants suffers heavy losses with pests and diseases. One of the most economically important diseases on squash is Phytophthora blight, caused by *Phytophthora capsici*. Control of this disease, so far, has largely depended on fungicides. Intensive use of fungicides leads to the appearance of less sensitive strains and resistant strains. The risk of fungicide resistance causes environmental hazards and decrease of fungicide efficacy. To prevent the excessive use of fungicides, an integrated approach is needed for the control of Phytophthora blight, including biological control along with limited fungicide use. PGPR strains have been demonstrated against a broad spectrum of pathogens on many vegetable crops. The overall aim of this dissertation was to investigate *Bacillus* PGPR strains for controlling Phytophthora blight on squash and plant defense induced by PGPR.

**Summaries**

In this dissertation, I was able to show that the application of selected *Bacillus* PGPR strains significantly increased plant growth and decreased Phytophthora blight disease severity on squash plants (Chapter 2). Of the efficient strains tested, *Bacillus subtilis* strain IN937b delayed disease development, enhanced the plant growth parameters, and increased leaf chlorophyll content in leaves under greenhouse conditions. Also, I established appropriate timing of IN937b applications with different plant ages to obtain remarkable efficacy of disease control. IN937b applications prior to the pathogen inoculation, offer adequate time or IN937b to multiply and establish on the root surface, which prevents early infection by *P. capsici*. 
Another important result of this dissertation is that I have described biofilm formation of selected *Bacillus* PGPR strains in the liquid or solid medium, and the production of surfactins by IN937b (Chapter 3). Use of specific PCR-primers was applied for the detection of genes involved in the biosynthesis of surfactins in *Bacillus* spp. The PCR-based screening has been a useful and efficient approach for the identification of potential surfactin producing bacteria, especially when the surfactin primers were designed to detect genes involved in the biosynthesis. The production of surfactins and biofilm suggests that biofilm may be important for rhizosphere colonization by IN937b. The population dynamics of IN937b were investigated in the commercial soilless media using serial dilutions and plating on selective media. Population densities stayed high at the early stage of inoculation e.g. 1 day after treatment, but decreased dramatically two weeks after treatment. IN937b also found in the interior tissues of the roots as they have naturally evolved to compete and survive in the harsh conditions and compete in space with the pathogen.

The antagonistic effects are mainly due to production of antimicrobial antibiotics, which play a major role in biological control of plant pathogens. The results from present study revealed the production of antifungal antibiotic subtilosin A from IN937b. The efficacy of antibiotics extracted from the culture of IN937b was evaluated for *in vitro* suppressing *P. capsici* *in vitro* (Chapter 4). The methanol extracts of IN937b inhibited the germination of the zoospores and sporangia formation. It may stop or delay zoospores entry into the host tissue at the site of infection, also influenced the development of *P. capsici* hypha and accumulated in squash roots. Inhibition of the pathogen is through the localized direct antifungal activity of IN937b and their cell-free
culture extracts, which points towards the activity of antibiotics as the potential mechanism of disease control.

Plant resistance against diseases can be systemically induced by plant pathogens or non-pathogenic rhizobacteria. IN937b was selected for further studies for its potential to induce systemic resistance in squash against *P. capsici* (Chapter 5). Systemic and local induction of resistance by PGPR was assessed through increased activities of plant defense related enzymes peroxidase (PO) and phenylalanine ammonia lyase (PAL) in squash plants treated with PGPR and challenged with *P. capsici*. To determine the existence of systemic resistance on squash plants, the pathogen *P. capsici* was inoculated as inducers on leaves spatially separated from the PGPR strain IN937b applied as a soil drench. The activities of PAL and PO were determined in bacterized roots and distant induced squash leaves. The results demonstrated that systemic resistance against the squash root disease was not only induced by plant pathogens, but also by non-pathogenic PGPR. When disease symptoms started to appear on the tissues, the enzyme activates reached a peak. This reduced pathogen infection, and impaired the disease development on squash roots. But the pathogen-induced enzyme activities was stronger than the PGPR-mediated ISR on squash plants. A split-root technique was utilized on squash plants grown in soilless potting mixes. IN937b stimulated PO activates in the bacterized side of roots locally and in the distant side of roots systematically. Without *P. capsici* infection, PAL activities were not influenced by IN937b locally and systematically.

**Future Perspectives**

Our data strongly support the hypothesis that PGPR strains provide remarkable disease suppression as an integrated approach in vegetable crops production. Further
studies need to be performed to evaluate the effect of PGPR in the field and to explain how plants communicate with PGPR in the soil.

In particular, the following issues might be taken into account in the future:

To extend our results of Chapter 2, I will try to optimize the biocontrol efficacy in the field including the rate of application, timing, number of applications, and bacterial survival at specific level of pH, temperature and soil fertility under field conditions. Furthermore, I will investigate the effect of PGPR combined with commonly used fungicides in the field to reduce the use of chemicals, which could be integrated into existing management programs for Phytophthora blight in squash.

Detection and visualization of biofilm formation in situ will be studied (Chapter 3). Visualization in situ act as a powerful tool that allows us to investigate the spatial colonization pattern of the pathogen and the biocontrol agent on the root surface, and also to determine biofilm production pattern of the biocontrol agent coincide with the spatial colonization of the pathogen. It will be interesting to characterize those signaling molecules released by the plant host that trigger Bacillus PGPR to colonize and form biofilm on the root surfaces, and to understand how the PGPR senses and responds to the signals in stimulating biofilm formation.

To expand the in vitro results in Chapter 4 and to study the hypothesized effects of subtilosin A, the mode of action of antifungal activity will be studied. As model systems to study the principles of antibiotics biosynthesis, Bacillus PGPR strains are natural producers of antibiotics that can be used to enhance antifungal activity. Based on the role of antibiotics and their action in the biocontrol of pathogens, antibiotics
produced by PGPR are excellent candidates for design and development of novel fungicides.

PGPR partly induced defense response in the squash plants. It suggested that signal substances initially form within inoculated sites, and translocate to the entire plant when plants were induced (Chapter 5). Certainly the signal would play an important role in connecting bacterized sites and distant sites of the plants. Surfactin, PAL and PO need to be investigated for their role at the molecular level in regulation of defense response by using real-time PCR.

It is known that diseases have deleterious effects on crop production that increasing the cost for growers and food safety. This study provides future researchers with important tools for understanding biocontrol mode of actions that can be used to develop powerful and effective biocontrol agents and to manipulate rhizosphere ecosystems for crop production.
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BIOGRAPHICAL SKETCH

Xiaodan Mo was born in Jinzu, Guangxi, China. She obtained her bachelor’s degree in the Department of Plant Protection from China Agricultural University in 2006, and she was admitted to the master of science program in the Department of Plant Pathology at China Agricultural University the same year. From 2006 to 2009, she was involved in the research project mainly focused on the function of β-glucanase and its heterologous expression in the *Bacillus* PGPR. In August 2009, Xiaodan was awarded a fellowship from China Scholarship Council and joined the Ph. D program in the Department of Plant Pathology at the University of Florida. In 2011, she began working with Dr. Shouan Zhang on biological control of Phytophthora blight by *Bacillus* PGPR strains on squash. As a graduate student, Xiaodan has presented her work in 2012 and 2013 American Phytopathological Society Annual Meetings. She received 2012 Warren Wood, Sr. Memorial Fellowship from Miami Dade County, FL. Xiaodan received her Ph. D. from the University of Florida in December 2013.