Selecting Plant Growth-Promoting Rhizobacteria (PGPR) for Both Biological Control of Multiple Plant Diseases and Plant Growth Promotion in the Presence of Pathogens

by

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Abstract

A study was conducted to select PGPR strains for broad-spectrum disease suppression and growth promotion in the presence of plant pathogens. First, 198 strains were tested for antibiosis capacity against nine different pathogens *in vitro*, including *Pythium ultimum, P. aphanidermatum*, three different isolates of *Rhizoctonia solani, Fusarium oxysporum* f. sp. *lycopersici* and f. sp. *vasinfectum*, *Xanthomonas campestris* pv. *vesicatoria* (Xcv), and *Pseudomonas syringae* pv. *tomato* (Pst). Thirty elite strains which inhibited 8 or 9 pathogens were then tested for traits often related to plant growth promotion, including N-fixation, IAA production, siderophore production, phosphate solubilization, phytase production, biofilm formation, and biosurfactant activity. All strains exhibited at least one trait of these traits. Second, these 30 PGPR strains were tested for biological control of four different plant diseases in the growth chamber. Five strains reduced the incidence or severity of 3 out of 4 tested diseases. AP69, AP199, AP200 significantly reduced two foliar bacterial diseases (Pst and Xcv) on tomato and *P. ultimum* on cucumber. AP197 and AP298 significantly reduced two foliar bacterial diseases on tomato (Pst and Xcv) and *R. solani* on pepper. Lastly, two separate experiments were conducted in the greenhouse, and each experiment included two individual PGPR strains and their mixtures, which were tested for biological control of three different diseases and for plant growth promotion in presence of pathogens. Mixtures exhibited better disease reduction and increases in growth (shoot dry weight and root dry weight) and root morphology parameters

(root volume, total root length, root surface area, and fine roots) compared with individual PGPR strains. In summary, selected individual PGPR strains and some mixtures exhibited both biological control of multiple plant diseases and plant growth promotion, and results were better with mixtures than with individual PGPR strains.

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List of Abbreviations

PGPR	Plant-Growth Promoting Rhizobacteria
PGP	Plant-Growth Promoting
TSA	Triptic Soy Agar
PDA	Potato Dextrose Agar
LB	Lysogeny Broth
IAA	Indole-3-Acetic Acid
UV	Ultraviolet
Trp	Tryptophan

Chapter I Literature Review

1. Introduction

Crop production is strongly impacted by plant diseases and by fertilization. Plant diseases cause yield losses and represent chronic threat to food security in many parts of the world. Current disease control methods are based primarily on chemical fungicides and bactericides as a reliable method for keeping economic stability of crop production. Chemical fertilizers are used to provide sufficient nutrients for optimizing crop yields. However, the exclusive reliance on the use of pesticides and chemically synthesized fertilizers often creates public concern around issues such as the development of pathogen resistance to pesticides, environmental pollution, contamination of surface and ground waters, and deleterious non-target effects on humans, beneficial soil microorganisms, insects, birds, and fish (Waard et al. 1993). Thus, public concern about the environment has increased the need to develop and implement alternative control approaches for crop protection. Using plant growth-promoting rhizobacteria (PGPR) as biopesticides and biofertilizers can be one such alternative (Banerjee et al. 2005; Chandler et al. 2011; Subba Rao 1993).

PGPR are root-colonizing bacteria that promote plant growth and often exhibit biological control of plant disease (Beneduzi et al. 2012; Kloepper and Schroth 1978). Some PGPR strains can enhance plant growth and yield, promote nutrient uptake, and biologically control plant disease (Kloepper et al. 1980b; Labuschagne et al. 2011; Liu et al. 2013). Successful colonization of the root is the first step for such PGPR beneficial effects (Choudhary and Johri 2009; Piromyou et al. 2011).

As agents for biological control, PGPR exhibit two major mechanisms of biological control. The first mechanism is a direct mode--antagonism (antibiosis, competition, and hyperparasitism)

in which the PGPR produce metabolites that directly affect the pathogen. The second mechanism is an indirect mode, termed induced systemic resistance, in which the PGPR triggers plant resistance against the pathogen. As agents for biofertilizers, PGPR promote plant growth by several mechanisms including by altering the microbial community structure in the rhizosphere, producing plant growth regulators (IAA, gibberellins, and cytokinins), producing volatile organic compounds, and exerting deleterious effects on other deleterious microorganisms. Although the positive effects of PGPR on plants usually are separated into two categories, biological control and growth promotion, there is a close relationship between plant growth promotion and biological disease control (Mariano and Kloepper 2000).

Typical biocontrol studies evaluate a single PGPR strain against a single pathogen (Murphy et al. 2000; Zhang et al. 2010). However, a single PGPR strain as biological control may suppress a only narrow range of pathogens, and exhibit inconsistent performance under environmental conditions. Therefore, mixtures of PGPR have been used to manage multiple plant diseases that often occur in the field (Domenech et al. 2006; Jetiyanon et al. 2003).

2. Plant Growth-promoting Rhizobacteria (PGPR) and colonization

2.1. Plant Growth-promoting Rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) are a group of beneficial bacteria that influence the growth, yield and nutrient uptake of plants, and that often exhibit biological control of plant disease (Kloepper and Schroth 1978; Udayashankar et al. 2011). The two main genera of PGPR strains include asporogenous fluorescent *Pseudomonas* spp. and species of *Bacillus*, and related Gram-positive spore-forming bacteria (Figueiredo et al. 2011). Although the preponderance of PGPR studies have been with fluorescent *Pseudomonas* spp., most commercially available

PGPR are bacilli (Brumm et al. 1991). This is because the dormant endospore of bacilli are tolerant to heat, desiccation, UV irradiation and organic solvents (Gates et al. 2010).

2.2. The process of colonization

PGPR influence plant growth and disease reduction by various direct or indirect mechanisms; however, successful colonization of the root is the first step leading to beneficial effects (Choudhary and Johri 2009; Piromyou et al. 2011). Thus, a thorough understanding of all steps involved in the root colonization by PGPR is required to improve the survival and competition of inoculated strains.

Bacterial root colonization (colonization process) is an active process whereby bacteria survive on seeds or plant parts, multiply in the spermosphere (region surrounding the seed) in response to seed exudates, attach to the root surface, and then grow on roots (Kloepper and Beauchamp 1992). The rhizosphere contains an abundant and highly active microflora because root exudates and rhizodeposits provide large amounts of easily degradable organic carbon sources (Campbell et al. 1990). Therefore, an important aspect of colonization is the ability to compete with indigenous microorganisms already present in the soil and rhizosphere of the inoculated plant (Schroth and Hancock 1982; Waard et al. 1993).

2.3. PGPR colonize different parts of plant

PGPR not only colonize the rhizosphere and rhizoplane, but some PGPR also can enter plants and colonize internal root and stem tissues as endophytes (Hallmann 2001; Kloepper et al. 1980a; Probanza et al. 2001). Many studies have focused on the colonization of beneficial bacteria in the rhizosphere since the 1980s. Approximately 10^7–10^9 CFU/g of cultivable rhizosphere bacteria were detected in rhizosphere soil, compared with 10^5–10^7 CFU/g on the rhizoplane (Benizri et al. 2001; Ugoji et al. 2005). The existence of endophytic colonization was proposed by 1887, but it

was not investigated due to the general belief that microorganisms detected inside healthy plants represented contamination (Smith 1911). Recent studies confirmed that the endophytes can enter roots, spread inside the plant, and colonize the stems, leaves, flowers and fruits (Hallmann 2001; Hardoim et al. 2008).

2.4. PGPR colonization traits

A variety of bacterial traits contribute to the colonization process, but only a few have been identified (Lugtenberg et al. 2001). These include motility, chemotaxis to seed and root exudates, production of pili or fimbriae, production of specific cell surface components, capacity to use specific components of root exudates and protein secretion, and quorum sensing (Barriuso et al. 2008; Dietel et al. 2013; Dutta and Podile 2010).

From the rhizosphere, PGPR move to root surfaces guided by chemotaxis, and facilitated by flagella (Compant et al. 2010; Compant et al. 2005). The root zone is considered to be a relatively rich source of carbon, because as much as 40% of plant photosynthates are transported to this zone (Kaitaniemi and Honkanen 1996). These carbon sources are secreted to the rhizosphere and rhizoplane as root exudates, and include amino acids, organic acids, and sugars (de Weert et al. 2004; Rudrappa et al. 2008). Root exudates provide rich nutrients for a diversity of microorganisms including the inoculated PGPR, which move to the nutrients (Walker et al. 2003). As an example, mutants of *P. fluorescens* defective in flagella-driven chemotaxis but retaining motility, exhibited strongly reduced root colonization, demonstrating that chemotaxis is an important competitive colonization trait (de Weert et al. 2002).

The O-antigen from lipopolysaccharides (LPS) has been shown to be necessary for efficient colonization of potato roots by *P. putida* and tomato root-tips by *P. fluorescens* WCS365 (Dekkers et al. 1998). However, the importance of LPS in colonization might be strain-

dependent since the O-antigenic side chain of *P. fluorescens* WCS374 did not contribute to potato root colonization (de Weger et al. 1989).

2.5. Techniques of detecting root colonization

Numerous methods for identifying root colonization by PGPR have been developed including traditional microbiology (Chowdhury and Dick 2012), immunology (Haverson et al. 2007), molecular insertion of specific marker genes (Franke et al. 2000), insertion of the green fluorescent protein (gfp) (Bloemberg and Lugtenberg 2001; Gilbertson et al. 2007), lacZ markers (Arsène et al. 1994), and fluorescent *in situ* hybridization (FISH) (Assmus et al. 1995; Bauman et al. 1980; Moter and Gobel 2000; Rothballer et al. 2003). Among these methods, FISH allows for direct visualization and characterization of the bacteria by providing information on the structure of the intact plant cell (Oliveira et al. 2009; Rothballer et al. 2003; Trejo et al. 2012). For a comprehensive understanding of root colonization, numerous methods should be combined together.

3. Biocontrol

PGPR exhibit several specific mechanisms of biological disease control, which can be grouped into two general mechanisms. The first is antagonism (antibiosis, competition, and hyperparasitism) in which the PGPR strain exerts its primary and direct action against the pathogen via antibiosis, or competition. Another mechanism is the indirect mode-induced systemic resistance in which PGPR trigger the resistance of plant against the pathogen (Compant et al. 2005; Kloepper et al. 2004).

3.1 The mechanism of antagonism

During the past two decades, the basis of antagonism as a biocontrol mechanism of PGPR has been extensively studied (Dowling and O'Gara 1994; Govindasamy et al. 2011). According to

Cook and Baker (1983), antagonism is defined as actively expressed opposition, and includes antibiosis, competition, and parasitism. Antibiosis appears to be the main mechanism by which most PGPR strains with biological control activity operate (El Meleigi et al. 2014; Fernando et al. 2006). A wide variety of PGPR metabolites, including antibiotics, siderophores, and cell wall degrading enzymes, are involved in biocontrol (Fernando et al. 2006; Jha and Subramanian 2014; Sayyed et al. 2013). Among these metabolites, antibiotics have been extensiely studied (Govindasamy et al. 2011).

3.1.1. Antibiotics

Antibiotics are low molecular weight organic compounds produced by microorganisms that inhibit or destroy the pathogen. Most antibiotics have been detected from the asporogenous *Pseudomonas* spp. Examples of such antibiotics include hydrogen cyanide (HCN) and 2,4-diacetylphloroglucinol (DAPG) by *Pseudomonas* sp. strain LBUM300 for biocontrol of bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*) on tomato (Lanteigne et al. 2012), phenazines by *P. aeruginosa* strain PNA1 for biocontrol of root rot (*Pythium myriotylum*) on cocoyam (Tambong and Hofte 2001), pyoluteorin by *P. putida* strain NH-50 for biological control of red rot (*Glomerellatucumensis*) on sugarcane (Hassan et al. 2011), pyrrolnitrin by *P. cepacia* strain B37 for biocontrol of dry rot (*Fusarium sambucinum*) on potato (Burkhead et al. 1994), 2-hexyl-5-propylresorcinol by *P. fluorescens* strain PCL1606 for biocontrol root rot (*Dematophora necatrix*) on avocado (Cazorla et al. 2006), d-gluconic acid by *Pseudomonas* strain AN5 for biocontrol of take-all (*Gaeumannomyces graminis* var. *tritici*) on wheat (Kaur et al. 2006), lipopeptides by *Pseudomonas* strain DF41 for biocontrol of Sclerotinia stem rot (*Sclerotinia sclerotiorum*) on canola (Berry et al. 2010), and phenazines and cyclic lipopeptides

by *Pseudomonas* strain CMR12a for biocontrol of root rot (*Rhizoctonia* spp.) on bean (D'aes et al. 2011).

In comparison to the fluorescent pseudomonads, *Bacillus* spp. produced substantially fewer antibiotics. Examples of anitbiotics produced by bacilli with biological control activity include zwittermicin A and kanosamine by *B. cereus* strain UW85 for biocontrol of damping-off (*Phytophthora medicaginis*) on alfalfa (Stabb et al. 1994), fengycin by *B. subtilis* strain F-29-3 for biocontrol of *Rhizoctonia* disease (Deleu et al. 2008), and iturin A by *B. amyloliquefaciens* strain B94 for biocontrol of *R. solani* (Yu et al. 2002).

3.1.2. Volatile compounds

A variety of volatile organic compounds (VOCs) have been shown to be produced by *Bacillus* spp. including 2, 3-butanediol, 2-ethyl-hexanol, 2, 4-bis (2-methyipropyl)-phenol, 4-hydroxybenzaldehyde, 2-nonanone, and various volatile blends. VOCs have been implicated in the biocontrol of postharvest decay (*Penicillium crustosum*) on citrus (Arrebola et al. 2010), inhibition of growth and spore germination of *F. oxysporum* f. sp. *cubense* (Yuan et al. 2012), inhibition of mycelial growth of *F. solani* (Li et al. 2015), induction of the systemic resistance to *Erwinia curotovora* subsp. *carotovora* (Ryu et al. 2004), and promotion of the growth of *Arabidopsis* (Ryu et al. 2003).

3.1.3. Lytic enzymes

Some PGPR strains excrete a high level of lytic enzymes, which play a vital role in their antifungal activity (Huang et al. 2005; Xiao et al. 2009). The enzymes chitinase and β-1,3-glucanase produced by *B. subtilis* strain EPCO 16 strongly inhibited *Fusarium. oxysporum* f. sp.*lycopersici* of tomato (Ramyabharathi et al. 2012). Similarly, chitinase produced by *B. cereus*

strain 28-9 was linked to biocontrol of Botrytis leaf blight (*Botrytis elliptica*) of lily (Huang et al. 2005).

3.1.4. Siderophores

Siderophores have also been recognized as an important antagonistic tool for some PGPR by binding iron with high specificity and affinity, making the iron unavailable for pathogens and limiting their growth (Gaonkar et al. 2012; Thomashow and Weller 1990). The siderophore of *B. subtilis* CAS15 was linked to biocontrol of Fusarium wilt (*F. oxysporum* Schl. f.sp. *capsici*) on pepper (Yu et al. 2011), and the siderophore of *Pseudomonas* spp. was linked to biocontrol of bacterial wilt (*Ralstonia solanaceraum*) on tomato (Jagadeesh et al. 2001).

3.1.5. Synergetic effects of PGPR metabolites

Synergy of different mechanisms produced by the same strain biological control of diseases, while one prominent biocontrol mechanism exhibited by a single strain. The extracellular enzyme (β-1, 3-glucanase) and an antibiotic that were produced by *B. subtilis* NSRS 89-24 played a synergistic role in the control of two fungal pathogens *Pyricularia grisea* and *R. solani* on rice (Leelasuphakul et al. 2006).

3.2. The mechanism of ISR

The reduction of severity or incidence of disease caused by host defense elicited by PGPR, which are spatially separated from pathogens is called induced systemic resistance (ISR) (Kloepper et al. 2004). Unlike direct biological control mechanisms, ISR results from PGPR triggering plant resistance to pathogens (Jellis 1998; Ramamoorthy et al. 2001; Vale et al. 2001).

3.2.1. ISR and SAR

The induced resistance triggered by a pathogen is often called systemic acquired resistance (SAR) (Durrant and Dong 2004; Ryals et al. 1996), while resistance triggered by PGPR is called

ISR (van Loon et al. 1998). SAR and ISR protect plants through different signaling pathways. Unlike SAR that is dependent on the salicylic acid (SA) pathway and causes visible symptoms, ISR is dependent on jasmonic acid (JA) and ethylene (ET) signaling in the plant and does not cause visible symptoms (Knoester et al. 1999; Maurhofer et al. 1998; Van der Ent et al. 2009).

3.2.2. Inducers of ISR

ISR is induced by metabolites or features of a specific bacterial strain (De Vleesschauwer and Höfte 2009). Over the past decade, a myriad of bacterial traits operative in triggering ISR have been identified, including cell structures such as flagella (Meziane et al. 2005), cell envelope components like lipopolysaccharides (Leeman et al. 1995), and metabolites including siderophores (Höfte and Bakker 2007; Press et al. 2001; Ran et al. 2005), N-alkylated benzylamine (Ongena et al. 2005), surfactin and fengycin lipopeptides (Ongena et al. 2007), VOCs (Ryu et al. 2004), phenolic compounds (Akram et al. 2013), signal molecules such as N-acyl-L-homoserine lactone (AHL) (Schuhegger et al. 2006). Among these inducers, VOCs may play a putative role in eliciting host defense and growth promotion (Ryu et al. 2004).

3.2.3. Defense mechanisms of ISR-mediated by PGPR

Physiology and metabolic responses are altered after the induction of ISR, leading to enhanced synthesis of some plant defense chemicals which limit the pathogen. PGPR cause a line of defense against pathogen spread in the plant, such as strengthening the epidermal and cortical cell walls as seen with *B. pumilus* strain SE34 in pea and tomato (Benhamou et al. 1996; Benhamou et al. 1998) and *P. fluorescens* WCS417r in tomato (Duijff et al. 1997). These biochemical or physiological changes are associated with the accumulation of pathogenesis-related proteins (PR proteins) and defense chemicals including phytoalexins, phenylalanine

ammonia lyase (PAL), and chalcone synthase (Dao et al. 2011; Mariutto et al. 2011; Ongena et al. 2000).

3.2.4. Broad-spectrum protection

Some PGPR strains have the potential to induce systemic resistance against multiple plant pathogens (Kloepper et al. 1997; Ramamoorthy et al. 2001). For example, PGPR strains *P. putida* 89B-27 and *S. marcescens* 90-166 both elicited ISR in cucumber against anthracnose caused by *Colletotrichum orbiculare* (Wei et al. 1991), Fusarium wilt caused by *F. oxysporum* f.sp. *cucumerinum* (Liu et al. 1995c), bacterial angular leaf spot caused *by P. syringae* pv. *lachrymans* (Liu et al. 1995a), cucurbit wilt infected by *E. tracheiphila* (Kloepper et al. 1997), and cucumber mosaic cucumovirus in cucumber and tomato (Raupach et al. 1996).

4. Growth promotion

PGPR have been shown to directly enhance plant growth by a variety of mechanisms, including biological nitrogen fixation, solubilization of mineral phosphate, secretion of plant hormones, and environment stress relief (Antoun and Prevost 2006; Lugtenberg and Kamilova 2009; Vessey 2003).

4.1. Biofertilizers

Biofertilizers are products containing living microorganisms that colonize the rhizosphere of plants subsequently increasing the supply or availability of primary nutrients and providing a growth stimulus to the target crop (Bhattacharjee and Dey 2014). The improvement of soil fertility is an essential strategy for increasing agriculture yield, and some microorganisms can develop this system by biological nitrogen fixation (Bhattacharjee et al. 2008) and phosphate solubilization (Gaur 1990).

4.1.1. Nitrogen fixation

Nitrogen (N) is the most vital nutrient for plant growth since it is required for biosynthesis of essential molecules such as amino acids and nucleic acids (Hewitt and Smith 1974; Wetzel and Likens 2000). Although approximately 78% of the atmosphere is nitrogen in the form of N_2, N_2 cannot be directly used by any organism (Delwiche 1970). However, nitrogen fixing microorganisms covert nitrogen gas (N_2) from the atmosphere into the plant-utilizable form, ammonian through the action of the nitrogenase-enzymatic complex during nitrogen fixation (Kim and Rees 1994).

Two groups of nitrogen fixing microorganisms have been extensively studied; symbiotic N_2 fixing bacteria *Rhizobium* (Zahran 2001) and *Bradyrhizobium* (Giraud et al. 2013; Sánchez et al. 2011), that are symbiotic with legumes and induce the formation of nodules, and non-symbiotic N_2 fixing bacteria such as *Azospirillum* (Fibach‐Paldi et al. 2012; Khammas et al. 1989), *Acetobacter* (James et al. 1994), *Bacillus* (Ding et al. 2005), and *Pseudomonas* (Yamanaka et al. 2005). PGPR present in the soil rhizosphere, rhizoplane and internal in plant tissues, have the capacity to fix nitrogen and increase the availability of nutrients in the rhizosphere (Adesemoye et al. 2010; Vessey 2003). In one experiment, a mixture of PGPR strains *B. amyloliquefaciens* IN937a and *B. pumilus* T4, supplemented with 75% of the recommended fertilizer was statistically equivalent to nutrient (nitrogen and phosphorus) uptake to the full fertilizer rate (Adesemoye et al. 2009).

4.1.2. Phosphate solubilization

Phosphorus (P) is an essential macronutrient for plant growth and development and exists in most agricultural soils largely in forms unavailable for plants due to its insolubility (Bray and Kurtz 1945; Malboobi et al. 2012). Although applying chemical fertilizer provides phosphorus

for plant use, a decrease in solubility and availability of phosphate results from the reaction of Ca in alkaline soils that form dibasic calcium phosphate dihydrate, octocalcium phosphate, and hydroxyapatite and by the reaction of Al and Fe in the acidic soils that form crystalline variscite (an Al phosphate) and strengite (an Fe phosphate) (Busman 1997). Each of these insoluble compounds of phosphate is generally not available to plants. Therefore, it is very important that insoluble phosphorus is converted into soluble phosphorus (HPO_4^{2-} or $H_2PO_4^-$) for plant uptake (Mengel et al. 2001). Phosphate solubilizing bacteria commonly exist in the rhizosphere, where they produce organic acids for solubilizing the inorganic mineral phosphate (Bolan et al. 1994) or enzymes such as phytases which release soluble phosphorus from organic compounds soil (Hayes et al. 2000). These processes facilitate the conversion of insoluble forms of P to plant-available forms (Rodriguez and Fraga 1999). Phosphate solubilizing bacterial most commonly belong to the genera *Azotobacter* (Kumar et al. 2001), *Rhizobium* (Sridevi and Mallaiah 2009), *Pseudomonas* (Selvakumar et al. 2009), and *Bacillus* (Maheswar and Sathiyavani 2012), which can enhance P uptake by the plant (Yu et al. 2012).

4.2. Secretion of phytohormones

Some PGPR strains produce phytohormones that stimulate plant growth, such as auxins, cytokinins, and gibberellins (Bottini et al. 2004; Garc á de Salamone et al. 2001; Khalid et al. 2004). The plant hormones ((indole-3-acetic acid (IAA), gibberellins, and cytokinins) are known to be involved in root initiation, cell division, and cell enlargement (Bottini et al. 2004). Among all the reported different types of phytohormones, production of IAA by PGPR has been recognized for a long time as a mode of action on the promotion of plant growth (Etesami et al. 2009). IAA-producing PGPR can increase root growth and root length, resulting in greater root surface area which enables the plant to access more nutrients from soil (Gilbertson et al. 2007).

4.3. Amelioration of abiotic stress

Agricultural crops are exposed to many stresses from both biotic and abiotic factors (Atkinson and Urwin 2012). These stresses decrease yields of crops and represent barriers to the introduction of crop plants into areas that are not suitable for crop cultivation (Rosielle and Hamblin 1981). PGPR strains containing 1-aminocyclopropane-1-carboxylate (ACC) deaminase have shown protection against stress via increaed growth (Grichko and Glick 2001; Nadeem et al. 2009; Shaharoona et al. 2006; Zahir et al. 2008; Zhang et al. 2008). Ethylene is an important phytohormone, but over-production of ethylene under stressful conditions can result in the inhibition of plant growth or even plant death, especially for seedlings (Beyer 1976). PGPR that produce ACC deaminase can hydrolyze ACC (the immediate precursor of ethylene) to alpha-ketobutarate and ammonia, and in this way promote plant growth (Saleem et al. 2007).

5. Biological control and Plant growth promotion by PGPR

5.1. Metabolites involved in both plant growth promotion and biological control

Some bacterial metabolites can cause both plant growth promotion and biological control, including siderophores, volatile organic compounds, and antibiotics. PGPR can promote plant growth by one or more different mechanisms (Glick 1995). Mechanisms include the direct mechanisms discussed above, and indirect mechanisms including inhibition of phytopathogens by the release of siderophores and volatiles (Gaonkar et al. 2012; Marques et al. 2010). Iron is an essential growth element for all living organisms, and intense competition is formed by the scarcity of iron. In the soil, most iron exists in an insoluble form (Fe^{3+}) under aerobic conditions, restricting its availability to plants and microbes (Dudeja et al. 1997). To meet their iron requirement, PGPR have evolved specific mechanisms to chelate insoluble iron through the release of siderophores and uptake of iron-siderophore complexes through specific outer

membrane receptor proteins. Sequestration of iron by siderophores produced by bacteria can deprive pathogenic fungi of Fe if fungi have a lower Fe affinity than the siderophore. For example, three siderophore-producing strains of endophytic *Streptomyces* were selected both for biocontrol of tomato early blight disease (*Alternaria alternata*) and for plant growth promotion (Verma et al. 2011). Ryu et al (2004; 2003) found volatile organic compounds of some *Bacillus* spp. promoted growth of Arabidopsis and tobacco in culture and induced systemic resistance toward *E. carotovora* subsp. *carotovora* in Arabidopsis.

5.2. Relationship between growth promotion and biological control

Although the beneficial effects of PGPR on plants usually are separated into two categories, biological control and growth promotion, there is a close relationship between them (Mariano and Kloepper 2000). PGPR promote the growth of the entire plant, which can result in the plant having increased tolerance to disease, and, conversely, biological control of plant diseases by PGPR may indirectly result in promotion of plant growth (Beneduzi et al. 2012). Hence, individual strains of PGPR have been shown to exhibit both growth promotion and biological control through various mechanisms (Wahyudi and Astuti 2011). In search of efficient PGPR strains, multiple traits related to plant growth promotion (PGP) and biocontrol activity have been tested together during the screening process, resulting in the identification of PGPR strains that exhibited multiple functions related to crop production (Ahmad et al. 2008; Praveen Kumar et al. 2014; Wahyudi and Astuti 2011).

6. Forming complex mixtures

6.1. Individual PGPR vs mixtures of PGPR

The majority of published reports of plant disease biocontrol evaluate single PGPR strains against a single pathogen through one main mechanism (Murphy et al. 2000; Zhang et al. 2010).

For example, Huang et al. (2012) reported that the antibiotic-producing bacterium *B. pumilis* strain SQR-N43 directly inhibited damping-off of cucumber, caused by *R. solani*. Hassan et al. (2010), similarly reports of antibiotic-producing rhizobacteria exhibiting biological control via antibiotic production have been reported with diverse bacteria in various host/pathogen systems, including *B. subtilis* strains NH-100 and NH-160 against red rot of sugarcane, caused by *C. falcatum* (Hassan et al. 2010); *B. subtilis* strains PFMRI, BS-DFS, and PF9 against bacterial wet rot of potato, caused by *R solanacearum* (Aliye et al. 2008) ; and *P. fluorescens* strain FP7 against mango anthracnose, caused by *C. gloeosporioides* (Vivekananthan et al. 2004).

Single PGPR strains with one main mechanism of action for biological control have also been selected based on production of siderophores and elicitation of induced systemic resistance (ISR). Yu et al. (2011) reported that the siderophore-producing *B. subtilis* strain CAS 15 competed for iron with the soilborne pathogen *F. oxysporum* Schl. f. sp. *capsici*, and also promoted growth of pepper (Yu et al. 2011). With ISR, *B. pumilus* strain SE34 induced defense to Fusarium wilt (*F. oxysporum*) (Benhamou et al. 1998) and late light (*P. infestans*) (Yan et al. 2002) in tomato. Similarly, *P. putida* 89B-27 and *S. marcescens* 90-166 induced systemic resistance in cucumber against cucumber anthracnose (*C. falcatum*), bacterial angular leaf spot (*P. syringae* pv. *lachrymans*) (Liu et al. 1995a), Fusarium wilt (*F. oxysporum* f. sp. *cucumerinum*) (Liu et al. 1995c), and cucumber mosaic cucumovirus (Raupach et al. 1996).

Despite the positive results reported in the preceding examples, single PGPR strains have not been used on a wide range of plant hosts and have typically exhibited inconsistent performance in the field for various reasons (Pal and Gardener 2006). First, a single PGPR strain typically does not have biological control activity against multiple pathogens. Second, a single strain is not likely to be active at a high enough level against pathogens under diverse conditions found in the

field, including competitive indigenous microorganisms, diverse environmental conditions, unpredictable weather, and multiple plant diseases (Elmqvist et al. 2003). The formulation of mixtures of PGPR is one strategy to address multiple modes of action and biocontrol of multiple pathogens (Domenech et al. 2006).

6.2. Broad-spectrum defense activity

Several studies have shown that compatible mixtures of PGPR strains can provide broad-spectrum activity against different pathogens. Ji et al (2006) used pairwise combinations of three foliar biological control agents and two selected PGPR strains against three foliar bacterial pathogens (*P. syringae* pv. *tomato*, *Xanthomonas campestris* pv. *vesicatoria* and *X. vesicatoria*) in tomato. Szczech and Dyśko (2008) mixed three different PGPR strains against two soil-borne disease (*F. oxysporum* f. sp. *radices-lycopersici* and *R. solani*) in tomato. Siddiqui and Shaukat (2002) used a mixture of PGPR against different types of pathogens that included a group of fungi (*Macrophomina phaseolina*, *F. solani*, and *R. solani*) and root-knot nematode (*Meloidogyne javanica*) in tomato. Raupach and Kloepper (1998) used two-way or three-way mixture against three different pathogens (*C. orbiculare*, *P. syringae* pv. *1achrymans*, *E. tracheiphila*) in a single host (cucumber). Jetiyanon et al (2003; 2002) evaluated pairwise combinations of seven PGPR strains against four different pathogens (*R.solanacearum*, *C. gloeosporioides*, *R. solani*, and *cucumber mosaic virus*) in four hosts (tomato, pepper, green kuang futsoi, and cucumber). All these tests were pre-screened in the greenhouse and worked effectively in the field.

6.3. Higher-level protection

Compatible mixtures of PGPR have also been shown to induce a higher-level of protection than individual PGPR strains. In a study of biocontrol of bacterial wilt of tomato, anthracnose of

pepper, damping off of green kuang futsoi, and cucumber mosaic virus, some PGPR mixtures caused at least a 50% disease suppression of most of these diseases compared to the non PGPR treated control treatment (Jetiyanon and Kloepper 2002). Mixtures of PGPR exhibited a general trend toward more consistent and higher magnitude disease suppression than did individual strains of PGPR (Bharathi et al. 2004; Lucas et al. 2009). In addition, some mixtures of PGPR, selected for elicitation of ISR, reduced disease at the same level as a commercially available chemical elicitor (Actigard ® Syngenta) (Raupach and Kloepper 1998).

6.4. Consistent performance

Compatible mixtures of PGPR can give consistent performance. Jetiyanon et al (2003) tested individual PGPR and mixtures in Thailand during the rainy season and winter season and showed that mixtures more consistently suppressed both disease severity and disease incidence in both seasons than did individual strains. Kim et al .(2008) demonstrated the efficacy of mixtures for controlling Phytophthora blight of pepper under two different field conditions with crop rotation.

6.5. Formulation of mixtures

The formulation of strain mixtures is a key approach to increase the efficacy of plant growth promotion and plant disease protection in the field (Choudhary and Johri 2009). Stable formulations using different carriers such as peat and talc have been developed for the delivery of the PGPR stains for field level application. Talc-based strain mixtures were effective against rice sheath blight and increased plant yield under field conditions greater than did individual strains (Nandakumar et al. 2001). Talc and peat based formulations of *P. chlororaphis* and *B. subtilis* were prepared and used for the management of turmeric rhizome rot (Nakkeeran et al. 2004).

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Chapter II Screening PGPR strains for biological control of multiple plant diseases and multiple plant growth promotion activities *in vitro*

Abstract

A study was designed to screen individual PGPR strains for *in vitro* broad-spectrum pathogen suppression and production of several physiological activities related to plant growth promotion. In all, 198 strains were tested for antibiosis capacity against nine different pathogens. These nine pathogens included *Pythium ultimum, P. aphanidermatum*, three isolates of *Rhizoctonia solani, Fusarium oxysporum* f. sp. *lycopersici, F. oxysporum* f. sp. *vasinfectum*, *Xanthomonas axonopodis* pv. *vesicatoria,* and *Pseudomonas syringae* pv. *tomato*. Thirty elite strains exhibited broad-spectrum biocontrol capacity that inhibited nine or eight different pathogens *in vitro*. In advanced antibiosis test, these strains were evaluated against major plant pathogens, including *P. ultimum*, *Phytophthora capsici*, *R. solani*, *F. virguliforme*, *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, and *P. syringae* pv. *tomato*. The same 30 strains were evaluated for traits related to plant growth promotion, including nitrogen fixation, phosphate solubilization, IAA production, siderophore production, biofilm formation, and biosurfactant activity. Among these 30 elite strains, 28 strains inhibited all seven tested pathogens and exhibited all of the tested plant growth promotion traits.

1. Introduction

Increasing public concern for green and sustainable agricultural has resulted in renewed interest in beneficial soil microorganisms that can improve soil quality and enhance plant health (Higa and Parr, 1994). Among these beneficial soil microorganisms, plant growth-promoting rhizobacteria (PGPR) have gained worldwide interest, because they can both promote plant growth and provide biological control of plant diseases (Saharan and Nehra, 2011). Using biopesticides or biofertilizers containing PGPR stains can be a way to reduce or supplement chemical pesticides or fertilizers (Kawalekar, 2013).

As agents for biological control, some PGPR strains exhibit a direct mode of biological control, that is antagonism in which PGPR strains exert their primary and direct action against the pathogen via metabolic products such as antibiotics (Beneduzi et al., 2012). Biological control using antagonistic PGPR strains has been demonstrated against many plant pathogens (e.g., *Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp., *Xanthomonas* spp., and *Pseudomonas* spp.) which have a wide host range, a cosmopolitan distribution, and the capacity to cause tremendous economic damage (Fatima et al., 2009; McCullagh et al., 1996; Sain, 2010; Yuan et al., 2013). However, most studies have focused on a single target pathogen.

As agents for plant growth promotion (PGP), PGPR often exhibit one or more common traits including nitrogen fixation, phosphate solubilization, production of plant growth regulators (indole-3-acetic acid (IAA), gibberellins, and cytokinins), or siderophore production. N-fixing bacteria can potentially increase nitrogen availability by converting nitrogen gas (N_2) into the plant-utilizable form of ammonia (Bhattacharjee et al., 2008). P-solubilizing bacteria can convert insoluble phosphorus in the soil into soluble phosphorus (HPO_4^{2-} or $H_2PO_4^-$) which can be taken up by the plant (Mengel et al., 2001). Production of IAA can increase root growth and root

length, resulting in greater root surface area which potentially enables the plant to access more soil nutrients (Gilbertson et al., 2007). Siderophores chelate iron, making it more available for plant growth and less available to pathogenic fungi, thereby limiting pathogen growth (Verma et al., 2011). Plant growth can potentially be improved by biofilm formation and biosurfactant production. Bacterial biofilms established on plant roots have been suggested to protect the colonization sites, increase nutrient uptake, and allow the plant to adapt to changing environmental conditions (Seneviratne et al., 2011; Weller and Thomashow, 1994). Biosurfactants produced by PGPR have been linked to improved soil quality via soil remediation, increasing the availability of nutrients for PGPR, and eliminating plant pathogens (Sachdev and Cameotra, 2013).

PGPR are known to influence disease reduction and plant growth; however, some strains that are effective *in vitro* or in the greenhouse may not be effective under field conditions. Various environmental factors may affect PGPR strains' growth and change their effects on the plant. PGPR strains that have broad-spectrum biocontrol activity and multiple plant growth promoting traits is a possible approach for allowing their adaptation to a complicated environment. The present study was designed to screen a large number of spore-forming bacilli strains for their broad-spectrum biocontrol activity and multiple PGP traits. Objectives of this study were to: 1) screen 198 PGPR strains for phenotypes related to biological control of multiple plant diseases, and 2) test 30 elite strains which showed broad-spectrum biocontrol activity for multiple traits related to growth promotion.

2. Materials and methods

2.1. Screening for antagonistic activity *in vitro*

2.1.1. PGPR strains, media and culture conditions

In this research, 198 PGPR strains known to have growth promotion and biocontrol potential were tested. The identity of all strains was determined using 16S rDNA sequencing with comparison to sequences of type strains. All strains used in this study were stored in tryptic soybean broth amended with 15% glycerol at -80 ℃. Each strain from ultra-cold storage was streaked onto tryptic soy agar (TSA) and then incubated at 28 ℃ for 48 h to check for purity. For preparing PGPR suspensions, a half –loop (10 µl) of bacteria was mixed with 9 ml sterilized water.

2.1.2. Screening for antagonistic activity *in vitro*

An antibiosis technique was developed in which different types of agar were used for the PGPR and the challenged pathogen. Antibiosis tests were conducted on PDA plates for fungal pathogens and on water agar plates for bacterial pathogens. Three holes of 13 mm diameter were made into the agar plate, and these were filled with melted tryptic soy agar (TSA). After cooling of the TSA, 10 µl of the PGPR suspension was applied to the TSA disc. Plates were incubated at 28 °C for 48 h, and then exposed to ultraviolet (UV) light (1000 x100 µJ /cm^2) for 2 min to prevent bacterial growth out of the TSA disc. For fungal pathogens, the test fungus (7 mm diameter) was taken from the edge of a growing culture, and placed in the center of a PDA plate. For bacterial pathogens, 5 µl of pathogen was mixed with 50 ml soft agar (0.4% agar in 50% TSB) cooled to 37 ℃. After gently swirling, 7 ml of the resulting suspension was added to each plate. Each plate contained two PGPR strains and a water control placed at approximately equal distance from each other at the edge of the plate. Plates were incubated at 28 ℃. The results were

evaluated for presence or absence of visual inhibition zones. Nine pathogens were tested, including two *Pythium* isolates (the causal agent of damping-off and root rot disease)- (*Pythium ultimum* and *Pythium aphanidermatum*); three *Rhizoctonia solani* isolates (the causal agent of damping-off disease) which were isolated from pepper, lettuce, and Zoysia grass; two *Fusarium oxysporum* isolates (the causal agent of wilt disease) – (*F. oxysporum* f. sp. *lycopersici and F. oxysporum* f. sp. *vasinfectum* which were isolated from tomato and cotton, respectively); one isolate of *Xanthomonas axonopodis* pv. *vesicatoria* (the causal agent of bacterial spot disease) which was isolated from tomato; and one isolate of *Pseudomonas syringae* pv. *tomato* (the causal agent of bacterial speck disease) which was isolated from tomato. For experimental use, *Pythium* spp. were grown on corn meal agar (CMA), and other fungal pathogens were grown on potato dextrose agar (PDA). *X. axonopodis* pv. *vesicatoria* was grown on yeast dextrose carbonate agar (YDC), and *P. syringae* pv. *tomato* was grown on King's B medium.

Strains inhibiting the growth of all 9 or 8 of the 9 pathogens were further evaluated for antibiosis against other plant pathogens, including *P. ultimum*, *Phytophthora capsici*, *R. solani*, *F. virguliforme*, *X. campestris* pv. *campestris*, *X. axonopodis* pv. *vesicatoria*, and *P. syringae* pv. *tomato*. Inhibition zones were measured from the edge of the PGPR strain to the pathogen at the day when the water control reached to the edge of the plate. The inhibition index was calculated using the formula of Vincent (1947):

$$I = \frac{C - T}{C} \times 100$$

Where, I=inhibition index, C=growth in control, T=growth in treatment.

The calculated inhibition indices were categorized as strong inhibition (+++) when I ≥20%, medium inhibition (++) when 10% ≤ I <20%, and weak inhibition (+) when I <10% (Wahyudi and Astuti, 2011). Each of these tests was repeated three times.

2.2. Detection of plant growth promotion traits of selected strains

Thirty elite strains which showed broad-spectrum antibiosis activities were chosen for testing traits related to plant growth promotion: nitrogen fixation, phosphate solubilization, IAA production, siderophore production, biofilm formation, and biosurfactant activity. The PGPR strains were purified on TSA agar for 2 days and then grown in Lysogeny Broth (LB) for 48 h. Each of these tests was repeated three times.

2.2.1. Nitrogen Fixation

PGPR strains were tested for their capacity to fix N using the nitrogen-free semisolid medium (JNFb) as described by Olivares, et al. (1996). Fifty microliters of PGPR culture was inoculated into 5 ml JNFb semisolid medium at 28 ℃. After incubation for 48 h at 28 ℃, the formation of veil-like pellicles was considered presumptive of nitrogen fixation.

2.2.2. Phosphate solubilization

Qualitative phosphate solubilization capacity was conducted by the plate assay using National Botanical Research Institute's phosphate growth medium (NBRIP) which contained calcium phosphate ($Ca_3(PO)_4$) as the inorganic source of phosphate (Nautiyal, 1999). Ten microliters of culture was dropped on the surface of medium, and the development of a clear zone around the culture indicated phosphate solubilization. Quantitative spectrophotometric assays of phosphate solubilizing activity were performed in three separate liquid NBRIP broth tubes, each containing a different P source ($Ca_3(PO)_4$, $FePO_4$, and $AlPO_4$) (Bashan et al., 2013). Presence of phosphorus

in solution was confirmed in the supernatant by the molybdate-blue method of Murphy and Riley (1962).

2.2.3. IAA production

IAA produced by bacteria was determined qualitatively and quantitatively by the colorimetrical assay (Gordon and Weber, 1951). Test bacterial strains were grown in modified Nutrient Broth-M26 for 24h with shaking (150 r/min) at 28 ℃, then 200 µl of a 24 hour nutrient broth culture was inoculated into 20 ml of minimal salt medium amended with 5 mM/L of L-tryptophan, and shaken for 48 h. After centrifuging at 12,000 rpm for 5 min, 1 ml of the supernatant was added to 2 ml $FeCl_3$-$HClO_4$ reagent in the dark. After 25 min, the development of a pink or red color indicated IAA production. IAA production was quantified by spectrophotometer (Thermo Spectronic Genesys 10S UV-Vis, Thermo Fischer Scientific Inc., Waltham, MA, USA) at an absorbance of 530 nm, and the concentration of IAA was determined in µg/ml using a standard curve.

2.2.4. Siderophore production

Siderophore production was qualitatively evaluated by chrome azurol S (CAS) medium (Alexander and Zuberer, 1991). Fresh PGPR strains were stabbed into the CAS medium with a sterilized toothpick and were incubated in the dark at 28 ℃ for 48 h. A yellow-orange halo around the bacteria indicated siderophore production.

2.2.5. Biofilm formation

Biofilm formation was qualitatively tested in biofilm growth medium (Hamon and Lazazzera, 2001; O'Toole et al., 1999). Fifty microliters of each PGPR culture were inoculated into 5 ml biofilm growth medium then incubated without agitation at 28 ℃ for 48 h. Formulation of a

pellicle on the surface medium indicated biofilm formation (Constantin, 2009; Morikawa et al., 2006).

2.2.6. Biosurfactant activity

Test strains were grown in 50 ml of biosurfactant production medium for three days at 28 ℃ in 250 ml Erlenmeyer flasks with constant shaking (130 r/min). This medium contained (per liter): K_2HPO_4, 9.30 g; KH_2PO_4, 4.50 g; $NaNO_3$, 2.50 g; KCl, 1.00 g; NaCl, 1.00 g; $MgSO_4 \cdot 7H_2O$, 0.40 g; $CaCl_2 \cdot H_2O$, 0.05 g; $FeCl_3$, 1.00 mg; $MnSO_4 \cdot 2H_2O$, 0.10 mg. Cultures were centrifuged at 6500 g for 20 min, and were filtered through 0.45 micron filters followed by 0.22 micron filters. Biosurfactant activity was detected by blood agar lysis, drop collapse, and emulsification stability (E_{24}). For the blood agar test, fresh PGPR strains were stabbed into the blood agar with a sterilized toothpick and were incubated at 28 ℃ for 48 h (Mulligan et al., 1984). A clear zone around the bacteria indicated biosurfactant production. For the drop collapse test, 7μl of motor oil (10w-40) was placed on a microscope slide. The slide was equilibrated for 1 h at room temperature, and then 20 μl of bacteria culture stained by methylene blue was added to the surface of the oil (Cipinyte et al., 2011; Youssef et al., 2004). Biosurfactant production was considered positive when the drop diameter was larger than that produced by distilled water as a negative control. E_{24} of culture samples was determined by adding 1 ml of kerosene to the same amount of culture, mixing with a vortex for 1 min and allowing to stand for 24 hours (Patel and Desai, 1997). The E_{24} index was calculated as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm):

$$E_{24} = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100$$

3. Results

3.1. Screening for antagonistic activity *in vitro*

Overall, over 65% of PGPR strains showed inhibition of three different isolates of *R. solani*; 55% of strains inhibited the growth of two different subspecies of *Fusarium oxysporum*; 44% of strains prevented the growth of *P. ultimum*; and < 30% of strains inhibited *P. aphanidermatum*, *X. axonopodis* pv. *vesicatoria*, and *P. syingae* pv. *tomato* (Table 1). Seven of these PGPR strains inhibited all 9 pathogens, and 23 strains inhibited 8 of 9 pathogens. Bacterial identification indicated that these PGPR strains belonged to four species in two genera: *Bacillus* (*B. amyloliquefaciens*, *B. stratosphericus*, and *B. altitudinis*), and *Paenibacillus* (*Paenibacillus peoriae*) (Table 2). These 30 elite strains were then moved to advanced antibiosis test.

Twenty-eight out of 30 strains produced inhibition zones to all seven tested pathogens (Table 3). However, no strain strongly inhibited all seven tested pathogens, due to medium or weak antibiosis to *P. ultimum*. Strain AP218 strongly inhibited all tested pathogens except *P. ultimum*. Strains AP52, AP198, AP199, AP200, AP212, AP213, and AP214 produced medium or strong inhibition to all 7 tested pathogens. Among these 30 tested strains, over strains showed strong inhibition to *P. capsici*, *R. solani*, *F. virguliforme*, *X. axonopodis* pv. *vesicatoria*, *X. campestris* pv. *campestris*, while 40% strains strongly inhibited *P. syringae* pv. *tomato*.

3.2. Detection of plant growth promotion traits of selected strains

3.2.1. Nitrogen Fixation

Presumptive nitrogen fixation was demonstrated by 29 (97%) of the isolates based on formation of a pellicle in the semi-solid free-nitrogen medium. All of these isolates were *Bacillus* spp. (Figure 2).

3.2.2. Phosphate solubilization

All of the tested strains grew in solid NBRIP medium using $Ca_3(PO)_4$ as the inorganic source of phosphorus; however, no halo was observed with any strains (Figure 3). In tests of these strains in liquid NBRIP medium with different phosphate sources as the inorganic source of phosphorus, strains solubilized 0.00-2.61 µg/ml $Ca_3(PO)_4$, 0.0-0.13 µg/ml $FePO_4$, and 0.00-1.67 µg/ml $AlPO_4$ (Table 4).

3.2.3. Indole acetic acid production

The appearance of a pink or red color in the solution after 25 minutes of reaction time indicated the existence of IAA (Figure 4). All the tested strains changed the color to pink or red, and were forwarded to produce IAA in media supplemented with tryptophan (Trp), at levels of 3.0 to 15.0 µg/ml (Table 4).

3.2.4. Siderophore production

The results were interpreted based on a change from blue to orange color due to transfer of the ferric ion by the siderophore (Figure 5). Twenty-nine of the 30 tested strains produced a yellow-orange halo around the bacterial colony. The one negative strain was *Paenibacillus* spp.

3.2.5. Biofilm Test

Twenty nine *Bacillus* strains formed a pellicle on the surface of the growth medium, which indicates biofilm formation.

3.2.6. Biosurfactant activity

Fourteen strains were positive in all three tests, including blood agar lysis, drop collapse, and emulsification stability (E_{24}).

4. Discussion

The results presented here demonstrate that some single PGPR strains exhibited both broad-spectrum biocontrol activity and production of multiple traits related to plant growth promotion. Among these 30 elite strains, 28 strains inhibited all seven tested pathogens in the advanced antibiosis test and produced all six tested traits related to plant growth promotion. Some PGPR strains have previously been reported to produce multiple traits related to PGP and biocontrol (Bakthavatchalu et al., 2012; Wahyudi and Astuti, 2011). However, the PGPR strains in these previous studies were all *Pseudomonas* spp. and were tested for antibiosis only against fungal pathogens. In contrast, in the current study, a total of 12 pathogens, belonging to six different genera, were used in preliminary and advanced antibiosis tests, and these included bacterial and fungal pathogens. In addition, the tested pathogens included foliar pathogens, vascular pathogens, and root pathogens. The results demonstrated that the elite PGPR strains had a broad antagonistic activity which could help in plant establishment and resisting against pathogens under field conditions. In these 30 elite strains, 29 strains belong to *Bacillus* spp. The two main genera of PGPR strains include asporogenous fluorescent *Pseudomonas* spp. and sporogenous *Bacillus* spp. (Figueiredo et al., 2011). Although there is a preponderant studies of PGPR for biological control and plant growth promotion are fluorescent *Pseudomonas* spp., sporogenous *Bacillus* spp. provide the probability, ability, and feasibility of commercialization (Brumm et al., 1991). This is because *Bacillus* spp. can form dormant endospores by the process of sporulation, and these spores are tolerant to heat, desiccation, UV irradiation and organic solvents (Gates et al., 2010).

All of the tested 198 spore-forming bacilli strains used in this study had previously demonstrated repeated biocontrol or growth promotion capacity in the PGPR lab of Auburn

University, and they were isolated from various locations and crops. Of the 198 strains tested, 186 strains belong to the *Bacillus* spp., and were separated into 15 different species, including *B. cereus* (37 strains), *B. simplex* (34 strains), *B. amyloliquefaciens* (30 strains), *B. megaterium* (25 strains), *B. stratosphericus* (15 strains), *B. safensis* (14 strains), *B. mycoides* (10 strains), *B. subtilis* (8 strains), *B. altitudinis* (4 strains), *B. solisalsi* (3 strains), *B. aerophilus* (1 strain), *B. mojavensis* (1 strain), *B. aerophilus* (1 strain), *B. nealsonii* (1 strain), *B. psychrosaccharolyticus* (1 strain), and *B. vireti* (1 strain). Of the 30 strains which inhibited 8 or 9 pathogens in the preliminary antibiosis test, 27 strains belong to *B. amyloliquefaciens*, while another three strains belong to *B. aerophilus*, *B. stratosphericus* and *Paenibacillus peoriae*, respectively. This demonstrated that *B. amyloliquefaciens* exhibited the best broad spectrum biocontrol activity when compared with other species in this collection. Various mechanisms have been attributed to the antagonistic activity, including production of bioactive compounds, quorum sensing and biofilm formation (Xu et al., 2013). *B. amyloliquefaciens* can produce different bioactive compounds at each phase of bacterial growth, such as surfactins at the log phase growth (Pathak et al., 2014), iturin A near the end of the log phase growth (Yu et al., 2002), and mycosubtilin during the transition between log phase growth and stationary phage growth (Arguelles-Arias et al., 2009). Therefore, *B. amyloliquefaciens* is widely recognized as a powerful biocontrol agent (Balhara et al., 2011).

The typical basis of selecting an antagonist is production of a clear inhibition zone against the pathogen on a solid growth. The potential antagonist should grow as well as the test pathogen on the solid medium for production of the antibiotic. However, selection of PGPR as antagonists is commonly conducted on Potato Dextrose Agar (PDA) which supports the growth of fungal pathogens better than PGPR strains (Goswami et al., 2014; Manivannan, 2012). Therefore, it is

necessary to develop an alternate method where the solid medium contains two different types of agar-- one for the PGPR and one for the challenged pathogen. In this agar plate assay, TSA was used for growth of the PGPR and PDA for growth of the fungal pathogens. Although the method of agar plate test for determining antagonistic potential provides a rapid and easy assay at the initial screening, some studies have demonstrated that inherent limitations are exist *in vitro* assay due to no relation between the size of inhibition zone and disease control in plants (Tolba and Soliman, 2013). Gupta et al (2010) documented the correlation between *in vitro* and *in planta*, while Ran et al (2005) did not. In the future, the biocontrol capacity of candidates with broad-spectrum biocontrol activity *in vitro* should be tested *in planta*.

Nitrogen (N) and phosphorus (P) are the two most common essential macro nutrients for plant growth and development (Hewitt and Smith, 1974). Growers rely on chemical fertilizers for increasing their yield. Due to environmental concern of excessive use of chemical fertilizers, there is renewed interest in biofertilizers. Some PGPR strains promote plant growth by acting as biofertilizers through nitrogen fixation or phosphate solubilization (Bhattacharjee and Dey, 2014). In our study, 29 of the 30 tested strains exhibited presumptive nitrogen fixation. Future works should examine nitrogenase activity to confirm this finding by gas chromatography using the C_2H_2 reduction technique (Hardy et al., 1973). In the P-solubilization test, no strains produced a halo on NBRIP medium. However, these strains could solubilize $Ca_3(PO)_4$, $FePO_4$, or $AlPO_4$ in broth. Some studies have demonstrated that some bacterial strains solubilize phosphates although they did not show any clear halos in the plate assay (Baig et al., 2010; Leyval and Berthelin, 1989; Louw and Webley, 1959; Mehta and Nautiyal, 2001). Thus, the quantitative method of phosphate solubilization in broth is more reliable than solubilization on agar. Most studies used $Ca_3(PO)_4$ as the phosphorus source for screening P-solubilizing bacteria

(Hayes et al., 2000; Selvakumar et al., 2009). However, $FePO_4$ and $AlPO_4$ are the most common P-sources in acidic soils. Therefore, using different P-sources would allow assessment of P-solubilization capacity in both alkaline and acidic soils.

IAA and siderophores are known to have dual roles in biological control and plant growth promotion. IAA can inhibit spore germination and mycelium growth of varioius pathogenic fungi (Brown and Hamilton, 1992). IAA is also involved in root initiation, cell division, and cell enlargement for growth promotion (Bottini et al., 2004). Iron can be used directly for plant growth, and sequestration of iron by siderophores produced by bacteria can deprive pathogenic fungi of Fe if fungi have a lower Fe affinity than the siderophore. For example, three siderophore-producing strains of endophytic *Streptomyces* were selected both for biocontrol of tomato early blight disease (*Alternaria alternata*) and for plant growth promotion (Verma et al., 2011). In the current study, all 30 tested PGPR strains were positive for IAA production, and 29 of these strains also produced siderophores.

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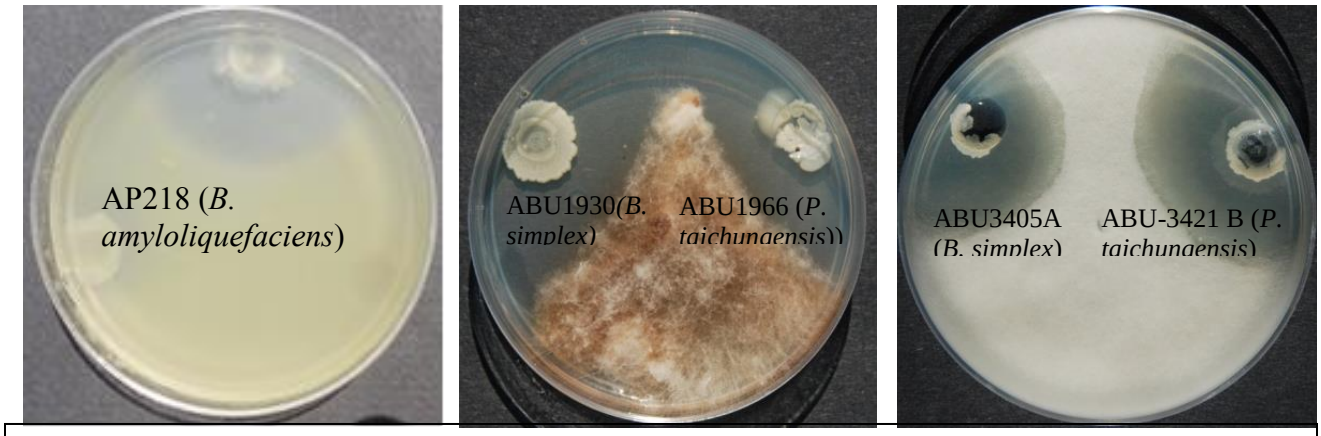
Table 1. Summary of preliminary antagonistic activity *in vitro*.

Name of Pathogens	Total	+[a]	%	–[b]	%
Pythium ultimum	198	88	44.4	110	55.6
Pythium aphanidermatum	198	55	27.8	143	72.2
Rhizoctonia solani （Pepper）	198	134	67.7	64	32.3
Rhizoctonia solani （Lettuce）	198	137	69.2	61	30.8
Rhizoctonia solani （Zoysia grass）	198	136	68.7	62	31.3
Fusarium oxysporum f. sp. *lycopersici*	198	109	55.1	89	44.9
Fusarium oxysporum f. sp. *vasinfectum*	198	107	54.0	91	46.0
Xanthomonas axonopodis pv. *vesicatoria*	198	52	26.3	146	73.7
Pseudomonas syingae pv. *tomato*	198	41	20.7	157	79.3

[a]+ = Positive for producing inhibition zone.

[b]– = Negative for producing inhibition zone.

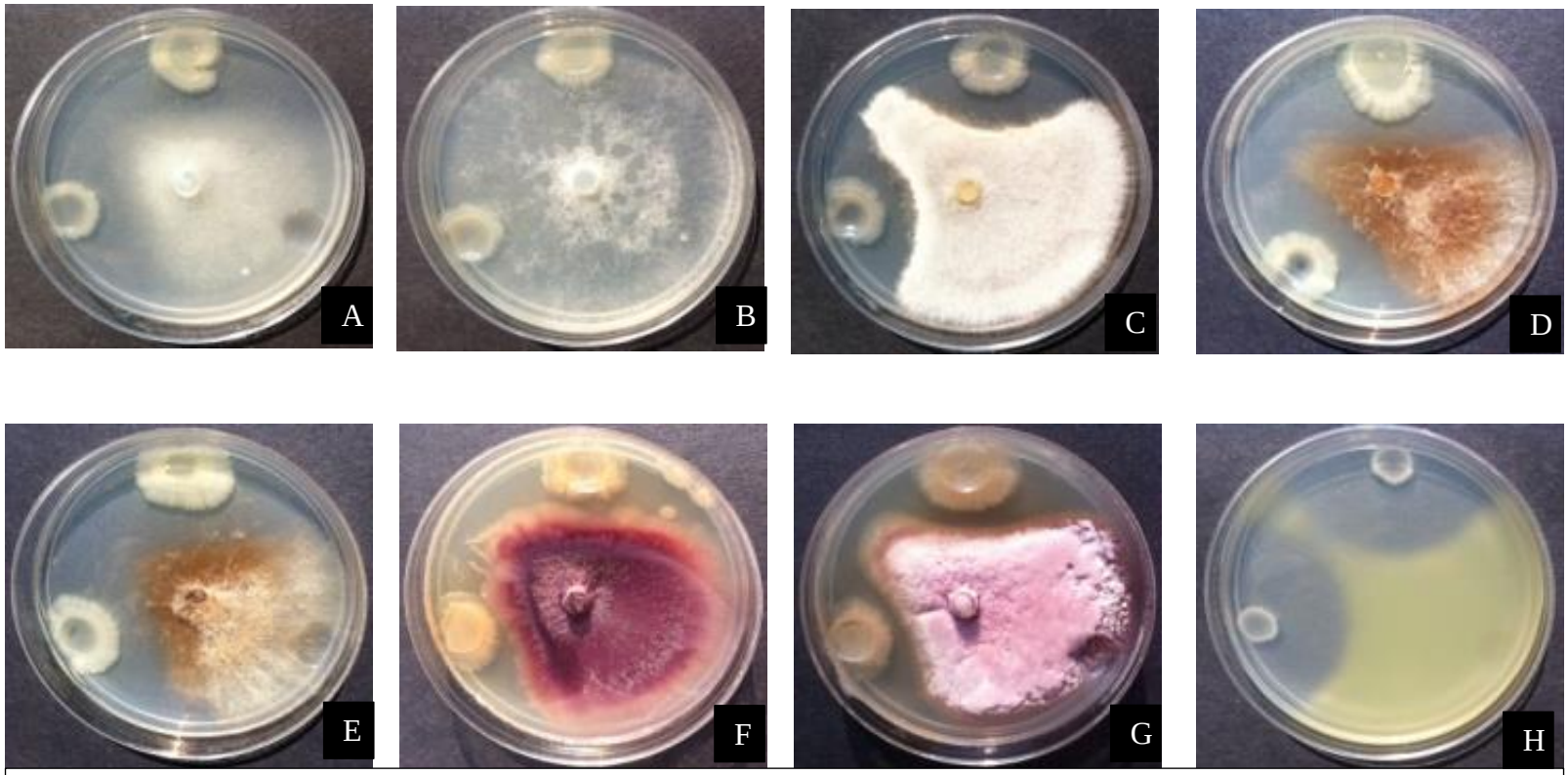


X. axonopodis pv. vesicatoria *R. solani* （Lettuce） *P. ultimun*

Table 2. Thirty elite strains which inhibited 8 or 9 pathogens in the preliminary screening.

Strains	ID	Pu[a]	Pa[a]	Rs-P[a]	Rs-L[a]	Rs-Z[a]	Fol[a]	Fov[a]	Xav[a]	Pst[a]
AP52	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	−	+
AP69	*Bacillus stratosphericu*	+	−	+	+	+	+	+	+	+
AP136	*Bacillus amyloliquefaciens*	+	−	+	+	+	+	+	+	+
AP188	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	−
AP194	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	−
AP195	*Bacillus amyloliquefaciens*	−	+	+	+	+	+	+	+	+
AP196	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	−	+
AP197	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	+
AP198	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	−	+
AP199	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	+
AP200	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	+
AP201	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	+
AP203	*Bacillus amyloliquefaciens*	+	−	+	+	+	+	+	+	+
AP208	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	−
AP210	*Bacillus amyloliquefaciens*	+	−	+	+	+	+	+	+	+
AP211	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	+
AP212	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	+
AP213	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	+
AP214	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	−
AP218	*Bacillus amyloliquefaciens*	+	−	+	+	+	+	+	+	+
AP241	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	−	+
AP294	*Paenibacillus peoriae*	+	−	+	+	+	+	+	+	+
AP295	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	−	+
AP296	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	−	+
AP297	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	−	+
AP298	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	−	+
AP301	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	−	+
AP305	*Bacillus amyloliquefaciens*	+	−	+	+	+	+	+	+	+
H57	*Bacillus amyloliquefaciens*	+	+	+	+	+	+	+	+	−
ABU2772	*Bacillus altitudinis*	+	−	+	+	+	+	+	+	+

[a]*Pythium ultimum*=Pu; *P. aphanidermatum*=Pa; *Rhizoctonia solani* (pepper) =Rs-P; *R. solani* (lettuce) = Rs-L; *R. solani* (zoysia grass) = Rs-Z; *Fusarium oxysporum* f. sp. *lycopersic*i= Fol; *Fusarium oxysporum* f. sp. *vasinfectum* =Fov; *Xanthomonas axonopodis* pv. *vesicatoria* =Xav; *Pseudomonas syringae* pv. *tomato* = Pst. + = Presence of inhibition zone, − = absence of inhibition zone.



H-57 (*B. amyloliquefaciens*) inhibited 8 different pathogens

A = *Pythium ultimum*, B = *P. aphanidermatum*, C= *Rhizoctonia solani* (Pepper), D = *R. solani* (Lettuce), E= *R. solani* (Zoysia grass), F = *Fusarium oxysporum* f. sp. *lycopersic*i, G= *F. oxysporum* f. sp. *vasinfectum* , H = *Xanthomonas axonopodis* pv. *vesicatoria*.

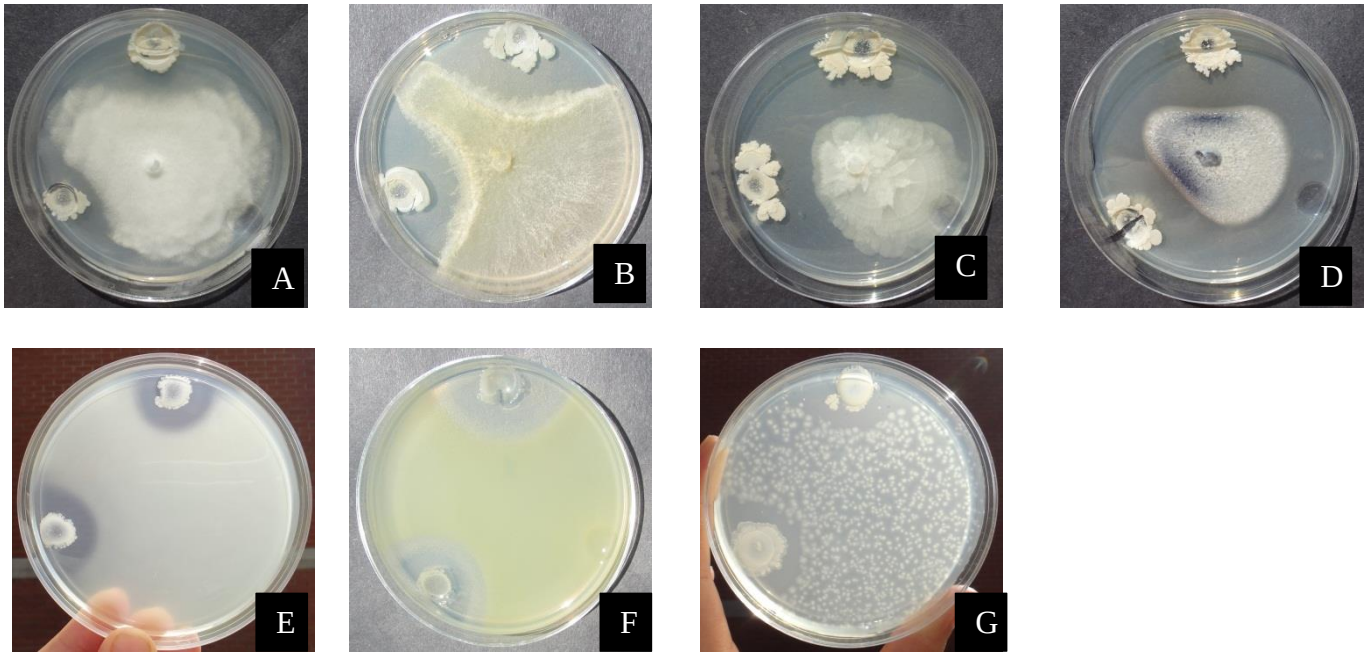
Table 3. Inhibition zone (mm) and inhibition index of 7 tested pathogens by 30 elite PGPR strains selected in the preliminary screening.

Strains	Pu[a]		Pc[a]		Rs[a]		Fv[a]		Xav[a]		Xcc[a]		Pst[a]	
	Zone[b]	Index	Zone[b]	Index	Zone[b]	Index	Zone[b]	Index	Zone[b]	Index	Zone[b]	Index	Zone[b]	Index
AP52	5.3	++	8.7	+++	11.7	+++	9.7	+++	16.0	+++	14.0	+++	6.7	++
AP69	1.0	+	12.0	+++	1.0	+	1.3	+	1.0	++	1.0	+	3.0	+
AP136	4.0	+	7.7	++	11.0	+++	9.3	+++	14.3	+++	11.7	+++	6.0	++
AP188	1.0	+	9.3	+++	9.3	+++	10.7	+++	10.0	+++	7.0	++	9.3	+++
AP194	1.0	+	5.7	++	7.3	++	10.0	+++	9.0	+++	9.0	+++	9.3	+++
AP195	1.3	+	6.0	++	9.0	+++	10.0	+++	13.7	+++	11.0	+++	6.0	++
AP196	3.0	+	11.3	+++	13.3	+++	13.0	+++	6.3	++	2.0	+	11.7	+++
AP197	3.0	+	7.7	++	9.7	+++	10.7	+++	10.0	+++	7.3	++	9.0	+++
AP198	6.3	++	11.0	+++	7.3	++	10.7	+++	15.7	+++	13.3	+++	8.0	++
AP199	5.0	++	11.3	+++	4.7	++	9.0	+++	16.3	+++	11.3	+++	8.3	+++
AP200	6.0	++	7.0	++	7.3	++	10.7	+++	11.7	+++	11.3	+++	6.0	++
AP201	3.0	+	8.3	+++	12.7	+++	8.0	++	10.0	+++	10.7	+++	4.7	++
AP203	2.3	+	11.0	+++	10.7	+++	7.7	++	9.7	+++	10.0	+++	4.3	+
AP208	2.3	+	11.0	+++	10.7	+++	10.3	+++	10.3	+++	10.7	+++	7.0	++
AP210	1.0	+	9.3	+++	11.3	+++	9.7	+++	12.7	+++	11.0	+++	6.3	++
AP211	2.0	+	12.3	+++	12.3	+++	10.7	+++	12.0	+++	9.0	+++	6.0	++
AP212	5.3	++	6.7	++	6.3	++	12.3	+++	10.3	+++	10.0	+++	4.7	++
AP213	5.7	++	9.0	+++	10.7	+++	12.0	+++	12.0	+++	10.7	+++	8.0	++
AP214	5.0	++	10.3	+++	12.0	+++	11.3	+++	12.3	+++	9.7	+++	7.3	++
AP218	1.3	+	9.0	+++	11.0	+++	10.7	+++	17.7	+++	10.7	+++	10.7	+++
AP241	1.7	+	9.0	+++	11.7	+++	10.0	+++	14.7	+++	12.7	+++	5.3	++
AP294	4.0	+	15.0	+++	7.0	++	3.3	+	1.7	++	2.0	+	0.0	–
AP295	2.7	+	7.3	++	10.7	+++	8.7	+++	21.3	+++	9.0	+++	8.3	+++
AP296	4.0	+	3.7	+	8.3	+++	9.0	+++	10.3	+++	6.0	++	3.7	+
AP297	2.7	+	7.0	++	9.7	+++	8.3	+++	15.0	+++	11.3	+++	12.7	+++
AP298	1.0	+	7.3	++	11.3	+++	10.3	+++	15.3	+++	14.3	+++	5.7	++
AP301	1.0	+	6.7	++	12.0	+++	9.7	+++	17.0	+++	14.3	+++	8.7	+++
AP305	1.3	+	9.7	+++	7.7	++	10.0	+++	19.3	+++	10.7	+++	12.0	+++
H57	1.0	+	7.0	++	8.3	+++	9.0	+++	15.0	+++	11.0	+++	14.0	+++
ABU2772	1.3	+	19.0	+++	11.7	+++	0.0	–	9.0	+++	3.0	+	14.0	+++

[a]*Pythium ultimum*=Pu; Phytophthora capsici=Pc; *Rhizoctonia solani* =Rs; *Fusarium virguliforme*= Fv; *Xanthomonas axonopodis* pv. *vesicatoria* =Xav; *Xanthomonas campestris* pv. *campestris*=Xcc, *Pseudomonas syringae* pv. *tomato* = Pst.

[b]The inhibition zone (mm) was measured from the edge of the PGPR strain to the pathogen when water control reached to the edge of the plate.

[c]The inhibition index was described as strong inhibition (+++) when inhibition index ≥20%, medium inhibition (++) when 10% ≤ inhibition index <20%, weak inhibition (+) when inhibition index < 10%.



The top strain was AP210 (*B. amyloliquefaciens*), left strain was AP211 (*B. amyloliquefaciens*), and the right was water control. A = *P. ultimum*, B = *R. solani*, C= *P. capsici*, D = *F. virguliform*, E = *X. axonopodis* pv. vesicatoria, F = *X. campestris* pv. campestris, G = *P. syringae* pv. tomato

Table 4. Plant growth promotion traits by 30 elite PGPR strains selected in the antagonistic activity *in vitro*.

Strains	N-fixation[a]	P-solubilization				Siderophore production[a]	IAA production (µg/ml)	Biofilm formation[a]	Biosurfactant activity		
		NBRIP (Medium)[a]	Ca (µg/ml)	Fe (µg/ml)	AL (µg/ml)				E_{24} (%)	Blood agar[b]	Drop collapse[a]
AP52	+	−	1.8	0.0	0.0	+	9.1	+	42.1	++	+
AP69	+	−	0.0	0.0	0.0	+	3.0	+	0.0	−	−
AP136	+	−	1.6	0.0	0.0	+	6.5	+	0.0	++	+
AP188	+	−	1.1	0.1	0.0	+	6.6	+	56.6	++	+
AP194	+	−	1.2	0.0	0.0	+	7.2	+	69.3	++	+
AP195	+	−	0.7	0.1	0.0	+	6.9	+	0.0	++	+
AP196	+	−	0.0	0.0	0.0	+	7.8	+	0.0	−	−
AP197	+	−	2.6	0.1	0.0	+	6.7	+	26.8	++	+
AP198	+	−	1.1	0.0	0.0	+	7.5	+	54.0	++	+
AP199	+	−	1.3	0.0	0.0	+	7.2	+	0.0	+	+
AP200	+	−	1.3	0.0	0.0	+	8.1	+	41.4	++	+
AP201	+	−	1.3	0.0	0.0	+	8.3	+	0.0	++	+
AP203	+	−	1.6	0.0	0.0	+	8.4	+	34.3	++	+
AP208	+	−	1.2	0.0	0.0	+	8.3	+	0.0	++	+
AP210	+	−	1.1	0.0	0.0	+	8.1	+	38.4	++	+
AP211	+	−	1.3	0.0	0.0	+	6.7	+	0.0	++	+
AP212	+	−	1.3	0.0	0.0	+	9.2	+	35.2	++	+
AP213	+	−	1.1	0.0	0.0	+	8.2	+	0.0	++	+
AP214	+	−	1.2	0.0	0.0	+	6.0	+	0.0	++	+
AP218	+	−	1.2	0.0	0.0	+	8.7	+	64.3	++	+
AP241	+	−	1.3	0.1	0.0	+	5.2	+	0.0	++	+
AP294	−	−	0.0	0.0	0.0	−	5.5	−	0.0	−	−
AP295	+	−	1.2	0.1	0.0	+	6.5	+	0.0	++	+
AP296	+	−	1.4	0.0	0.0	+	6.3	+	0.0	++	+
AP297	+	−	1.2	0.0	0.0	+	9.5	+	0.0	++	+
AP298	+	−	1.2	0.0	0.0	+	8.7	+	74.3	+	+
AP301	+	−	1.3	0.0	0.0	+	8.2	+	0.0	++	+
AP305	+	−	1.3	0.0	0.0	+	15.0	+	74.5	+++	+
H57	+	−	0.1	0.1	1.7	+	8.6	−	42.6	++	+
ABU2772	+	−	0.0	0.0	0.0	+	6.4	+	0.0	−	−

[a]+ = Positive for presumptive N-fixation, P-solubilization on NBRIP medium, siderophore production, biofilm formation, or drop collapse, − = Negative for presumptive N-fixation, P-solubilization on NBRIP medium, siderophore production, biofilm formation, or drop collapse.

[b]The diameter of the clear zones depends on the concentration of the biosurfactant. The zones of clearing were scored as follows: '−', no hemolysis; '+', incomplete hemolysis; '++', complete hemolysis with a diameter of lysis < 1 cm; '+++', complete hemolysis with a diameter of lysis ≥ 1cm.

Figure 1. Antibiosis evaluation scale in the preliminary screening.

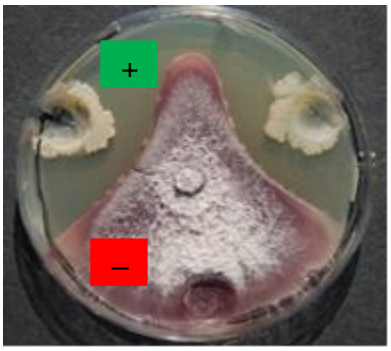
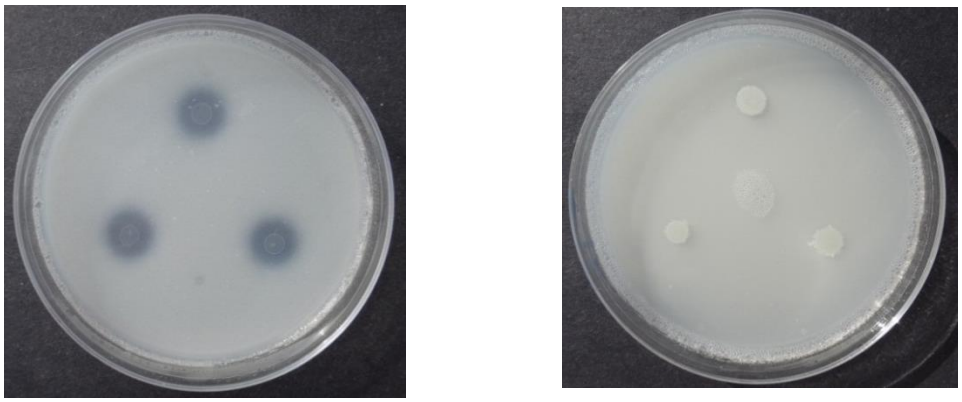


Figure 2. Growth pellicle formation by presumable N-fixing isolates (arrows) in semi-solid N-free culture medium. (A) Positive, strain AP52 (*B. amyloliquefaciens*), (B) Negative, strain AP176 (*P. amylolyticus*).

A (Positive) B (Negative)

Figure 3 Development of clear zone around the PGPR strain in the NBRIP medium. (A) Positive, strain P106 (*Pseudomonas tremae*), (B) Negative, strain AP213 (*Bacillus amyloliquefaciens*).

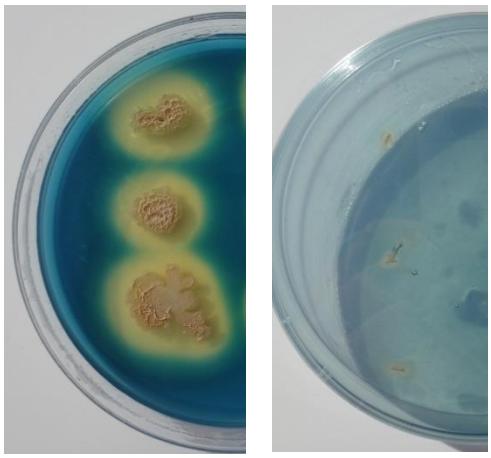


A (Positive) B (Negative)

Figure 4. The appearance of a pink or red color in the solution after 25 minutes reaction time indicated the existence of IAA. (A) Positive, red color, strain AP305 (*B. amyloliquefaciens*), (B) Positive, pink color, strain AP218 (*B. amyloliquefaciens*), (C) Negative, yellow, medium control.

A (Positive-Red) B (Positive-Pink) C (Negative-Yellow)

Figure 5. Development of yellow-orange halo around the PGPR strain in the CAS medium. (A) Positive, strain AP241 (*B. amyloliquefaciens*), (B) Negative, strain P151 (*P. jamilae*).



A (Positive) B (Negative)

Chapter III Selecting individual PGPR for biological control of multiple plant diseases in the growth chamber

Abstract

A study was designed to screen individual PGPR strains for providing broad-spectrum disease suppression. Thirty plant growth-promoting rhizobacteria **(**PGPR) strains selected *in vitro* for broad-spectrum biocontrol activity and multiple traits related to growth promotion were tested for biological control of multiple plant diseases *in vivo*. The specific diseases and hosts test in this study included bacteria spot of tomato caused by *Xanthomonas campestris* pv. vesicatoria, bacterial speck of tomato caused by *Pseudomonas syringae* pv. tomato, damping-off of pepper caused by *Rhizoctonia solani*, and damping-off of cucumber caused by *Pythium ultimum*. No individual PGPR strains significantly reduced all four tested diseases. Five individual PGPR strains out of the thirty tested strains inhibited three out four tested diseases and nineteen individual PGPR strains reduced two out of four diseases.

1. Introduction

Biological control using plant growth-promoting rhizobacteria (PGPR) strains exhibiting antagonism against plant pathogens has been demonstrated to be effective for managing some plant diseases in the greenhouse and field (Beneduzi et al., 2012). Research of PGPR for biocontrol typically begins by assessing the efficacy of single PGPR strains against multiple plant pathogens *in vitro* (Köberl et al., 2013; Kumar et al., 2012) and then of a single PGPR strain to a single plant disease *in vivo* (Li et al., 2011; Liu et al., 2009). However, several different diseases often occur in a single crop or different crops in field conditions. Therefore, there is a need to select PGPR strains with the capacity to control multiple plant diseases *in vivo*. Microorganisms as biological control agents typically have a relatively narrow spectrum of activity (Baker, 1991). Typically, a single PGPR strain does not have disease reducing activity against multiple plant diseases. One exception to this is when the mechanism for disease protection is induced systemic resistance (ISR) (Kloepper et al., 2004; Kloepper et al., 1997). However, antagonism is the main mechanism for achieving biological control in fruit and vegetable crops (Droby et al., 1989; Sharma et al., 2009). Selecting individual PGPR strains that exhibit both broad-spectrum biocontrol activities *in vitro* for antagonism against multiple plant diseases *in vivo* offers an alternative concept to control multiple plant diseases by ISR.

In the current study, two soilborne fungal diseases and two foliar bacterial diseases were tested. Seedling disease, commonly called "damping off," can be caused by a number of soil-borne fungi such as *Pythium spp*., *Rhizoctonia solani*, *Phytophthora capsici*, and *Fusarium spp*. These pathogens infect a large number of vegetables and agronomic crops. Damping off occurs when seeds or young seedlings are attacked by these pathogens. Pre-emergence damping-off occurs when infected seeds fail to germinate, while post-emergence damping-off occurs when the

infected seedlings collapse. Bacterial spot, caused by *Xanthomonas campestris* pv. vesicatoria, and bacterial speck, caused by *Pseudomonas syringae*pv pv. tomato, are among the most economically important diseases of tomato (Jones et al., 1991). Although these bacterial diseases are not always present at the destructive level, the loss of marketable yield can reach 43% in under optimal conditions (Pernezny et al., 1996). The development of bacterial spot is favored by warm temperature (24-30 ℃) and high precipitation, while bacterial speck is favored by low temperature (18-24 ℃) and high moisture (Jones et al., 1991). The lesions occur on leaves, and spread to stem and fruit, and cause the yield loss of marketable fruit.

The objective of this study was to select individual PGPR strains *in vitro* with broad-spectrum biocontrol activity and multiple traits related to growth promotion for biological control of multiple plant diseases *in vivo*.

2. Materials and Methods

2.1. PGPR cultural

Thirty stains selected *in vitro* were tested in four groups (group-A, group-B, group-C and group-D); two groups with 8 stains, and another two groups with 7 strains. In each experiment, treatments (single PGPR strains, a non-bacterized but pathogen-challenged disease control, and a non-treated healthy control) were arranged in a randomized complete block design (RCBD) with 6 replications. Each PGPR group was separately tested for four different plant diseases, and conducted twice in the growth chamber. The specific diseases and hosts tested in this study included bacteria spot of tomato caused by *Xanthomonas campestris* pv. vesicatoria (Xcv), bacteria speck of tomato caused by *Pseudomonas syringae* pv. tomato (Pst), damping-off of pepper caused by *Rhizoctonia solani* (Rs), and damping-off of cucumber caused by *Pythium ultimum* (Pu). Inoculum of PGPR was grown on tryptic soy agar (TSA) at 28 ℃ for 48 h and a

single colony was transferred to tryptic soy broth (TSB) and incubated with shaking (150 rpm) at 28 ℃ for 48 h. The bacterial culture was centrifuged at 3500 rpm for 15 min and the pellet was resuspended in sterile distilled water to 10^6 CFU/ml.

2.2. Pathogens and culture conditions

The pathogenic fungi *R. solani* (R1) and *P. ultimum* (Py13), were maintained on Corn Meal Agar (CMA) slants at room temperature for long-term storage. *R. solani* were transferred onto PDA plates and grown for 5 days for experimental use. Millet seed inoculum of *P. ultimum* was prepared as described by Howell (2007). Granules of *P. ultimum* inoculum were ground by a coffee grinder and stored in an autoclaved jar at room temperature. The bacteria, Xcv 95A. 213 and Pst 95A. 514, were maintained in TSB supplemented with 20% glycerol at −80 °C and were transferred to TSA plates for experimental use. A single colony was transferred to TSB and incubated with shaking (150 rpm) at 28 ℃ for 48 h. Bacterial cultures were centrifuged at 3500 rpm for 15 min, and the pellet was resuspended in sterile distilled water to 10^7 CFU/ml.

2.3. Growth chamber study

Four mycelial plugs (7 mm) of *R. solani* from the edge of pre-grown cultural or 1/8 tsp (0.62 ml) of *P. ultimum* inoculum were mixed into 100 cc of commercial potting substrate (Sunshine mix, Sun Gro Horticulture, Agawam, Maine). Five seeds of pepper or cucumber were placed into the Magenta GA-7 plant culture box (77 mm × 77 mm × 97 mm) 24 h after of pathogen inoculation. One ml of PGPR suspension (10^6 CFU/ml) was applied to each seed prior to covering with 50 ml potting medium and the lid. The culture boxes were incubated in a growth chamber at 24°C with a 12-h photoperiod. The number of seeds emerged at 14 or 7 days and the number of seedlings surviving at 21 or 14 days after sowing the seed were recorded for *R. solani*

and *P. ultimum* respectively. Incidence of pre-emergence damping off and of post-emergence damping off was calculated using the formula:

$$\text{Pre} - \text{emergence damping off} = \frac{\text{The number of non} - \text{emerged seeds}}{\text{The number of sown seeds}} \times 100\%$$

$$\text{Post} - \text{emergence damping off} = \frac{\text{The number of dead seedlings}}{\text{The number of emerged seedlings}} \times 100\%$$

Tomato seedlings were grown in germination trays containing 25 cm^2 holes for three weeks, and then transplanted into the plant culture box (77 mm × 77 mm × 97 mm) filled with 200 ml potting substrate. One week after transplanting, plants were sprayed with PGPR suspension (10^6 CFU/ml) and sterile distilled water through foliar application. Plants were then covered with the same plant culture box to retain humid conditions, and put into the growth chamber at 24°C with a 12-h photoperiod. Three days after spraying with PGPR, plants were challenge-inoculated with Xcv or Pst by spraying the whole plant and were covered again by the box. Plants were returned to the growth chamber, and the covered box was removed 4 days after inoculation. Plants were watered daily. The disease severity was assessed at 7 days after pathogen challenge for Xcv and 10 days after challenge for Pst. In the rating of disease, four compound leafs were chosen from the bottom of the whole plant, and the disease severities of compound leafs were averaged by the disease severity of leaflets on that compound leaf. The leaflet was rated using a 0-4 rating scale, where 0=healthy leaflet, 1= <20% necrotic area of leaflet, 2 = 20-50% necrotic area of leaflet, 3= 51-80% necrotic area of leaflet, 4 = 80-100% necrotic area of leaflet or fully dead leaflet.

2.4. Statistical analysis

All data were analyzed by analysis of variance (ANOVA), and the treatment means were separated by Fisher's protected least significant difference (LSD) test at P=0.05 using SAS 9.4 software (SAS Institute, Cary, NC, USA).

3. Results

3.1. *Rhizoctonia solani* damping-off of pepper

There was no significant suppression of the incidence of pre-emergence damping-off with any of the PGPR strains (Table 1). Three out of 30 tested stains significantly decreased the disease incidence of post-emergence damping off ($P \leq 0.05$), when compared with the disease control: stain AP197 from group-A, strain AP298 from group-C, and strain AP208 from group-D. The incidence of post-emergence damping with these PGPR treatments ranged from 13.8% to 23.1%, while post-emergence damping off's incidence of disease control ranged from 33.2% to 43.0%.

3.2. *Pythium ultimum* damping-off of cucumber

There was no significant suppression of the incidence of pre-emergence damping-off with any of the PGPR strains (Table 2). Five of 30 tested stains significantly decreased the disease incidence of post-emergence damping off ($P \leq 0.05$), when compared with the disease control: stains AP69, AP136, AP199, and AP200 from group-A and stain AP196 from group-C. The post-emergence damping-off' s incidence of these PGPR treatments ranged from 52.6% to 60.3%, while post-emergence damping-off' s incidence of disease control plants ranged from 85.0% to 91.7%.

3.3. Bacterial spot disease

Twenty six of 30 tested stains provided significant disease suppression ($P \leq 0.05$) against *X. campestris* pv. *vesicatora*, when compared with the disease control (Table 3): all the tested strains in the group-A and group-B; strains AP52, AP241, AP298, AP301 and ABU2772 from group-C; strains AP188, AP194, AP198, AP214, AP296 and H57 from group-D. The disease severity resulting from treatment with these PGPR treatments ranged from 1.23 to 1.83, while disease severity of disease control ranged from 2.04 to 2.22.

3.4. Bacterial speck of tomato

Twenty six of 30 tested stains provided significant disease suppression ($P \leq 0.05$) against *P. syringae* pv. *tomato*, when compared to the disease control treatment (Table 4): strains AP69, AP195, AP197, AP199, AP200, AP201, and AP203 from group-A; all the tested strains in the group-B; strains AP52, AP297, AP298, AP301 and ABU2772 from group-C; strains AP188, AP194, AP198, AP208, AP214, AP295 and AP296 from group-D. The disease severity of these PGPR treatments ranged from 0.49 to 1.42, while disease severity of disease control ranged from 1.23 to 1.73.

3.5. Broad-spectrum biocontrol activity

Overall, in sixteen experiments repeated twice, no individual PGPR strains significantly decreased these four different diseases (Table 5). Five individual PGPR stains inhibited three out four tested diseases: treatments AP197 and AP298 significantly reduced two foliar bacterial diseases on tomato and *R. solani* on pepper, and treatments AP69, AP199, and AP200 significantly reduced two foliar bacterial diseases on tomato and *P. ultimum* on cucumber. Nineteen individual PGPR strains reduced two out of four diseases: treatments AP52, AP188, AP194, AP195, AP198, AP201, AP203, AP210, AP211, AP212, AP213, AP214, AP218, AP294, AP301, AP305 and ABU2772 significantly reduced two foliar bacterial diseases on tomato; treatment AP136 significantly reduced *P. ultimum* on cucumber and *X. campestris pv. vesicatora* on tomato, treatment AP208 significantly reduced *R. solani* on pepper and *P. syringae pv. tomato* on tomato (Table 5).

Means of disease reductions of two foliar bacterial diseases and *R. solani* were 41.3% and 30.6% for strain AP197 and AP298 respectively (Table 6). Among these three PGPR strains for biocontrol two foliar bacterial diseases and *P. ultimum*, strains AP69, AP199 showed slightly

greater level of disease suppression as compared with strain AP200. Means of disease reductions were 34.8% and 29.9% for strains AP69 and AP199 respectively, and 10.2% for AP200. Four individual PGPR stains (AP69, AP197, AP199, and AP298) out of 30 tested strains showed the best broad-spectrum to biocontrol multiple plant diseases.

4. Discussion

The results presented here demonstrate that some individual PGPR strains can provide biological control against multiple plant diseases on different hosts through the mechanism of antagonism. Specifically, from the growth chamber trials, five individual PGPR stains significantly inhibited three of the four tested diseases, and 19 individual PGPR strains inhibited two of the four tested diseases. These results are in agreement with previous studies in which individual PGPR strains *P. putida* 89B-27 and *S. marcescens* 90-166 exhibited biological control of anthracnose caused by *Colletotrichum orbiculare* (Wei et al., 1991), Fusarium wilt caused by *F. oxysporum* f.sp. *cucumerinum* (Liu et al., 1995b), bacterial angular leaf spot caused *by P. syringae* pv. *lachrymans* (Liu et al., 1995a), cucurbit wilt caused by *E. tracheiphila* (Kloepper et al., 1997). However, the mechanism by which strains 89B-27 and 90-166 provide biological control was ISR, in which PGPR activate plant defenses which can lead to protection against multiple plant diseases (Zehnder et al., 2001), while antagonism was used in the current study. A significant amount of research on antagonism has been conducted in the phytopathogens of major crops (Beneduzi et al., 2012; Liu et al., 2009), fruits (Droby et al., 1989; Li et al., 2011) and vegetables (Pernezny et al., 1996; Sharma et al., 2009). Biological control agents which can control more than one pathogen are extremely interesting, making them ideal inoculants for crop production (Berg et al., 2001). In most studies, the antagonist was selected against a single target pathogen, causing the individual antagonists results in control of only one or two diseases *in vivo*

(Baker and Cook, 1974). Some antagonists have been defined as broad-spectrum candidates based only on *in vitro* tests (Köberl et al., 2013; Kumar et al., 2012; Wahyudi and Astuti, 2011). Our data suggest that antagonism is also an effective way for individual PGPR strain against multiple plant diseases as occurs with ISR. Broad-spectrum biocontrol activity which was initially screened by 12 different pathogens *in vitro* for these selected strains may explain why individual PGPR strain provided disease suppression to multiple plant diseases *in vivo*.

Five individual PGPR stains (AP69, AP197, AP199, AP200, and AP298) reduced the disease severity of bacteria rot and bacteria speck through foliar application, and also reduced the disease incidence of post-damping off of cucumber or pepper through seed treatment. Effective colonization by PGPR strains has been demonstrated to contribute to the suppression plant pathogens (Demoz and Korsten, 2006; Huang et al., 2012). The results of our study suggest that the same PGPR strains can survive in both in the rhizosphere and phyllosphere, because disease protection occurred against both soilborne and foliar pathogens. Similar finding was reported in previous studies showing that *P. fluorescens* strain Pf7-14 was applied as a seed treatment and foliar spay to biological control of rice blast (Krishnamurthy and Gnanamanickam, 1998) and *P. fluorescens* strain Pf1 was applied on the rhizosphere and phyllpsphere to control the late leaf spot of groundnut (Meena, 2010). Difference between our research and previous studies are *Bacillus* spp. strains were used in the current study and the same PGPR strain colonized on the different host. Phyllosphere and rhizosphere colonization are different colonization process, and phyllosphere colonization is more difficult due to the limitation of nutrients and opening to a dynamic envirnment (Andrews, 1992). Therefore, PGPR are most often used as seed treatments on crops to against soilborne fungi and nematodes by the successful colonization of rhizosphere (Abbasi et al., 2011; Hashem and Abo-Elyousr, 2011; Sikora, 1992), but suggested as foliar

application to control foliar diseases by multiple foliar application or integrating with the rhizosphere-applied PGPR (Ji et al., 2006). However, it is possible that biological control could result from PGPR without extensive colonization, especially if pre-formed metabolites are included in the PGPR preparation applied to plants. More extensive studies are needed to characterize the rhizosphere and phyloplane colonization patterns for the five individual PGPR strains selected in the current study.

The wide defense range of the selected five individual PGPR strains to three pathogens is striking, indicating a complicated mechanism of action that may involve more than one metabolite as suggested previously (Howell, 1982; Lumsden and Locke, 1989). Many different bacterial metabolites have been reported to cause antagonism (Deleu et al., 2008; Dowling and O'Gara, 1994; Fernando et al., 2006; Lanteigne et al., 2012). Some metabolites such as surfactins, iturins and fengycins showed a wide antagonistic activity to against bacteria (Monteiro et al., 2005), fungi (Deleu et al., 2008) and nematode (Mutua et al., 2011), while others such as cell wall degrading lytic enzymes (Huang et al., 2005) and siderophores (Yu et al., 2011) are specific to control fungal diseases. In our study, some individual PGPR could both control of fungal disease and bacterial disease. However, the specific metabolites involved in control are not known. Future work will focus on the identification of the metabolites responsible for the observed biocontrol activity to different types of pathogen.

Twenty six PGPR strains exhibited biological control activity to each bacterial disease while only three PGPR strains exhibited biological control activity to *R. solani* and 5 strains to *P. ultimum*. This may be explained by the application order of PGPR and pathogen. In tests with bacterial diseases, the aim is to control diseases that are spread through rain and irrigation. Therefore, PGPR were applied three days before the pathogen. Various defense mechanisms may

combined together to increase the activity of disease suppression. First, PGPR can occupy the same niche and use the limited nutrient source on the leaf as the pathogen (dos Passos et al., 2014). Second, PGPR may produce antibacterial compounds before pathogen challenged. In the future, it is very interesting to apply pathogen before PGPR since these two bacterial pathogens are seedborne diseases. In the soilborne diseases tests, planting seed and inoculation of pathogen was one day earlier than the inoculation of PGPR increased the challenge of biological control. In the previous studies, PGPR were applied at the same time of pathogen inoculation (Elazzazy et al., 2012) or 2 days earlier (Huang et al., 2012). In the current study, no individual PGPR strains could control two soilborne diseases. This range of activity suggests a complex mechanism of action that might apply to one pathogen but not the other. This result is in agreement with Lumsden and Locke (1989), who found that 20 isolates of *Gliocladium virens* varied in their efficacy in controlling *P. ultimum* and *R. solani*, and that some isolates controlled *R. solani* but not *P. ultimum*, and vice versa. Harris (1999) used *Truchoderma koningii* to control *R. solani* and *P. ultimum*, but it was not consistent as the fungicides. In the future, mixing various effective strains for each soilborne disease may provide the potential to control both diseases.

Effective individual PGPR strains did not show significant disease suppression in the incidence of pre-emergence damping-off, while they were effective in controlling post-emergence damping off. This can be explained by the pre inoculation of pathogen. In contrast, Howell (2007) tested a number of *Trichoderma* spp, which effective in controlling pre-emergence damping-off of *Pythium* spp. and *Rhizopus oryzae*, but not post-emergence damping-off.

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Table 1. Protection of Pepper from pre-emergence damping-off and post-emergence damping-off caused by *Rhizoctonia solani* in growth chambers

Group-A [a]			Group-B [a]			Group-C [a]			Group-D [a]		
Strain [b]	Pre [c]	Post [d]	Strain [b]	Pre [c]	Post [d]	Strain [b]	Pre [c]	Post [d]	Strain [b]	Pre [c]	Post [d]
Disease	25.0 a	36.3 a	Disease	36.7 ab	58.3 ab	Disease	36.7 a	33.2 a	Disease	30.0 ab	43.0 a
AP69	28.3 a	30.8 ab	AP210	43.3 ab	34.8 b	AP52	28.9 a	29.5 ab	AP-188	23.3 ab	43.3 a
AP136	26.7 a	44.4 a	AP211	51.7 a	76.8 a	AP196	27.8 a	30.6 ab	AP-194	33.3 a	36.6 ab
AP195	29.2 a	37.3 a	AP212	43.3 ab	35.8 b	AP241	27.8 a	23.8 ab	AP-198	26.7 ab	30.2 ab
AP197	23.3 a	16.8 bc	AP213	30.0 b	52.8 ab	AP297	23.3 a	24.7 ab	AP-208	18.9 b	23.1 b
AP199	26.7 a	42.5 a	AP218	41.7 ab	46.7 b	AP298	26.7 a	13.8 bc	AP-214	35.6 a	40.6 ab
AP200	25.8 a	44.2 a	AP294	43.3 ab	50.0 b	AP301	26.7 a	29.4 ab	AP-295	31.1 ab	31.9 ab
AP201	28.3 a	30.4 ab	AP305	51.7 a	44.7 b	ABU2772	32.2 a	34.1 a	AP-296	35.6 a	38.4 ab
AP203	27.5 a	34.0 ab	–	–	–	–	–	–	H57	24.4 ab	38.1 ab
Healthy	2.5 b	0.0 c	Healthy	0.0 c	0.0 c	Healthy	0.0 b	2.2 c	Healthy	1.1 c	0.0 c
$LSD_{0.05}$	12.9	17.7	$LSD_{0.05}$	19.9	25.7	$LSD_{0.05}$	13.9	33.2	$LSD_{0.05}$	12.4	18.8

[a]The experimental design was a randomized complete block with six replications per treatment. Experiments were repeated twice. Values were means of two experiments. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]Five seeds were sowed in each replication. Pathogen was inoculated 24 hours before sowing the seed, and PGPR was dropped 1.0 ml (10^6 CFU/ml) per seed.

[c]Pre= Incidence of pre-emergence damping-off. Values were determined 14 days after sowing the seed. Pre-emergence damping-off = (The number of non-emerged seeds) / (The number of sown seeds) ×100%.

[d]Post= Incidence of post-emergence damping=off. Values were determined 21 days after sowing the seed. Post-emergence damping-off = (The number of dead seedlings) / (The number of emerged seedlings) ×100%.

Table 2. Protection of Cucumber from pre-emergence damping-off and post-emergence damping-off caused by *Pythium ultimum* in growth chambers

Group-A [a]			Group-B [a]			Group-C [a]			Group-D [a]		
Strain [b]	Pre [c]	Post [d]	Strain [b]	Pre [c]	Post [d]	Strain [b]	Pre [c]	Post [d]	Strain [b]	Pre [c]	Post [d]
Disease	26.7 a	91.7 a	Disease	36.7 ab	60.8 a	Disease	25.0 b	85.0 a	Disease	26.7 abc	66.7 a
AP69	28.3 a	60.2 bc	AP210	30.0 ab	39.2 a	AP52	38.3 ab	72.9 ab	AP-188	30.0 abc	52.1 a
AP136	43.3 a	60.3 bc	AP211	21.7 bc	35.0 a	AP196	25.0 b	52.6 b	AP-194	21.7 bc	58.2 a
AP195	35.0 a	90.9 ab	AP212	26.7 abc	37.5 a	AP241	30.0 ab	54.8 ab	AP-198	38.3 a	75.0 a
AP197	41.7 a	73.6 abc	AP213	33.3 ab	44.2 a	AP297	30.0 ab	83.3 ab	AP-208	21.7 bc	54.3 a
AP199	26.7 a	54.2 c	AP218	36.7 ab	33.6 a	AP298	26.7 b	79.2 ab	AP-214	28.3 abc	80.5 a
AP200	40.0 a	53.0 c	AP294	40.0 a	39.6 a	AP301	43.3 a	81.8 ab	AP-295	33.3 ab	51.4 a
AP201	40.0 a	75.0 abc	AP305	31.7 ab	48.3 a	ABU2772	33.3 ab	63.6 ab	AP-296	30.0 abc	51.3 a
AP203	28.3 a	79.0 abc	–	–	–	–	–	–	H57	16.7 c	60.2 a
Healthy	3.3 b	0.0 d	Healthy	10.0 c	0.0 b	Healthy	5.0 c	0.0 c	Healthy	1.7 d	0.0 b
$LSD_{0.05}$	17.4	30.8	$LSD_{0.05}$	17.6	28.2	$LSD_{0.05}$	14.3	32.2	$LSD_{0.05}$	14.8	29.2

[a]The experimental design was a randomized complete block with six replications per treatment. Experiments were repeated twice. Values were means of two experiments. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]Five seeds were sowed in each replication. Pathogen was inoculated 24 hours before sowing the seed, and PGPR was dropped 1.0 ml (10^6 CFU/ml) per seed.

[c]Pre= Incidence of pre-emergence damping-off. Values were determined 7 days after sowing the seed. Pre-emergence damping-off = (The number of non-emerged seeds) / (The number of sown seeds) ×100%.

[d]Post= Incidence of post-emergence damping=off. Values were determined 14 days after sowing the seed. Post-emergence damping-off = (The number of dead seedlings) / (The number of emerged seedlings) ×100%.

Table 3. Protection of tomato from black rot caused by *Xanthomonas campestris pv.* vesicatora in growth chambers

Group-A [a]		Group-B [a]		Group-C [a]		Group-D [a]	
Strain [b]	Disease Severity [c]	Strain [b]	Disease Severity [c]	Strain [b]	Disease Severity [c]	Strain [b]	Disease Severity [c]
Disease	2.22 a	Disease	2.22 a	Disease	2.11 a	Disease	2.04 a
AP69	1.23 c	AP210	1.83 b	AP52	1.83 bc	AP-188	1.57 cd
AP136	1.42 bc	AP211	1.57 c	AP196	1.97 ab	AP-194	1.56 cd
AP195	1.67 b	AP212	1.69 bc	AP241	1.83 bc	AP-198	1.50 d
AP197	1.32 c	AP213	1.67 bc	AP297	2.18 a	AP-208	1.82 abc
AP199	1.42 bc	AP218	1.63 bc	AP298	1.64 cd	AP-214	1.68 bcd
AP200	1.65 b	AP294	1.48 c	AP301	1.63 cd	AP-295	1.88 ab
AP201	1.69 b	AP305	1.52 c	ABU2772	1.47 d	AP-296	1.71 bcd
AP203	1.65 b	–	–	–	–	H57	1.59 bcd
Healthy	0.00 d	Healthy	0 d	Healthy	0 e	Healthy	0.00 e
$LSD_{0.05}$	0.28	$LSD_{0.05}$	0.26	$LSD_{0.05}$	0.27	$LSD_{0.05}$	0.32

[a]The experimental design was a randomized complete block with six replications per treatment. Experiments were repeated twice. Values were means of two experiments. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]One plant was in each replication. Plants were sprayed with PGPR suspension (10^6 CFU/ml) one week after transplanting, and were challenge-inoculated with pathogen solutions (10^7 CFU/ml) three days after inoculating PGPR.

[c]Harvest and disease severity rating were assessed at 7 days after posting pathogen challenge. In the rating of disease, four compound leafs were chosen from the bottom of the whole plant, and the disease severity of compound leafs were averaged by the disease severity of leaflets on that compound leaf. The leaflet was rated using the 0-4 rating scale, where 0=Healthy leaflet, 1= <20% necrotic area of leaflet, 2 = 20-50% necrotic area of leaflet, 3= 51-80 % necrotic area of leaflet, 4 = 80-100 % necrotic area of leaflet, or fully dead leaflet.

Table 4. Protection of tomato from black speck caused by *Pseudomonas syringae pv.* tomato in growth chambers

Group-A [a]		Group-B [a]		Group-C [a]		Group-D [a]	
Strain [b]	Disease Severity [c]	Strain [b]	Disease Severity [c]	Strain [b]	Disease Severity [c]	Strain [b]	Disease Severity [c]
Disease	1.45 a	Disease	1.73 a	Disease	1.23 a	Disease	1.36 a
AP69	0.49 f	AP210	1.21 c	AP52	0.82 d	AP-188	0.86 d
AP136	1.22 ab	AP211	1.30 bc	AP196	1.14 ab	AP-194	0.97 cd
AP195	0.61 ef	AP212	1.42 b	AP241	1.14 ab	AP-198	0.80 d
AP197	0.52 f	AP213	1.38 bv	AP297	0.99 bc	AP-208	0.87 cd
AP199	0.83 de	AP218	1.31 bc	AP298	1.05 bc	AP-214	0.93 cd
AP200	1.12 bc	AP294	1.28 bc	AP301	0.96 c	AP-295	0.85 cd
AP201	0.91 cde	AP305	1.37 bc	ABU2772	1.04 bc	AP-296	1.18 cd
AP203	1.07 bcd	–	–	–	–	H57	1.04 ab
Healthy	0.00 g	Healthy	0 d	Healthy	0 e	Healthy	0.00 bc
$LSD_{0.05}$	0.3	$LSD_{0.05}$	0.18	$LSD_{0.05}$	0.17	$LSD_{0.05}$	0.22

[a]The experimental design was a randomized complete block with six replications per treatment. Experiments were repeated twice. Values were means of two experiments. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]One plant was in each replication. Plants were sprayed with PGPR suspension (10^6 CFU/ml) one week after transplanting, and were challenge-inoculated with pathogen solutions (10^7 CFU/ml) three days after inoculating PGPR.

[c]Harvest and disease severity rating were assessed at 10 days after posting pathogen challenge. In the rating of disease, four compound leafs were chosen from the bottom of the whole plant, and the disease severity of compound leafs were averaged by the disease severity of leaflets on that compound leaf. The leaflet was rated using the 0-4 rating scale, where 0=Healthy leaflet, 1= <20% necrotic area of leaflet, 2 = 20-50% necrotic area of leaflet, 3= 51-80 % necrotic area of leaflet, 4 = 80-100 % necrotic area of leaflet, or fully dead leaflet.

Table 5. List of plant growth-promoting rhizobacteria (PGPR) strains showing broad-spectrum capacity.

Rs+Pu+Xcv+Pst [a]	Rs +Xcv+Pst	Pu +Xcv+Pst	Xcv+Pst	Pu+Xcv	Rs+Pst
(0 strain)	AP197 and AP298 (2 stains)	AP69, AP199, and AP200. (3 strains)	AP52, AP188, AP194, AP195, AP198, AP201, AP203, AP210, AP211, AP212, AP213, AP214, AP218, AP294, AP301, AP305 and ABU2772. (17 strains)	AP136 (1 strain)	AP208 (1 strain)

[a]Rs=*Rhizoctonia solani*, Pu=*Pythium ultimum*, Xcv= *Xanthomonas campestris pv.* vesicatora, and Pst= *Pseudomonas syringe* pv. tomato.

Table 6 . Summary of suppression of three different pathogens by individual plant growth-promoting rhizobacteria (PGPR) strains over two repeated trials

	Mean percentage of disease reduction (%)						
Strain	Rs-Pre [a]	Rs-Post [b]	Pu-Pre [c]	Pu-Post [d]	Xcv [e]	Pst [f]	Total Mean
AP197	6.8	53.7	−	−	40.5	64.1	41.3
AP298	27.2	58.4	−	−	22.3	14.6	30.6
AP69	–	–	− 6.0	34.4	44.6	66.2	34.8
AP199	–	–	0.0	40.9	36.0	42.8	29.9
AP200	–	–	− 49.8	42.2	25.7	22.8	10.2

[a]Rs-Pre=Pre-emergence damping-off of *Rhizoctonia solani*.
[b]Rs-Post=Post-emergence damping-off of *Rhizoctonia solani*.
[c]Pu-Pre= Pre-emergence damping-off of *Pythium ultimum*.
[d]Pu-Post= Post-emergence damping-off of *Pythium ultimum*.
[e]Xcv= *Xanthomonas campestris pv.* vesicatora.
[f]Pst= *Pseudomonas syringe* pv. tomato.

Chapter IV Mixtures of Plant Growth-Promoting Rhizobacteria enhance biological control of multiple plant diseases and plant growth promotion in the presence of pathogens

Abstract

Two separate experiments were conducted in the greenhouse, and each experiment included two individual PGPR strains and their mixtures, which were tested for their potential biological control of three different diseases and for plant growth promotion in presence of pathogens. Mixtures exhibited better disease reduction and increases in growth (shoot dry weight and root dry weight) and root morphology (root volume, total root length, root surface area, average diameter, and fine roots) compared with individual PGPR strains. In summary, the tested individual PGPR strains and their mixtures exhibited both biological control of multiple plant diseases and plant growth promotion, and results were better with mixtures than with individual PGPR strains.

1. Introduction

In response to public health concerns, there is a renewed interest in beneficial soil microorganisms to reduce or supplement chemical pesticides or fertilizers (Ahemad and Kibret, 2014; Bhattacharyya and Jha, 2012). Several studies have demonstrated that growth-promoting rhizobacteria (PGPR) could provide biological control of plant diseases (Beneduzi et al., 2012) and promote plant growth (Saharan and Nehra, 2011).

Several studies have shown that mixtures of PGPR could enhance biological control activity for multiple plant diseases through induced systemic resistance (Jetiyanon and Kloepper, 2002; Raupach and Kloepper, 1998), and of a single plant disease through antagonism (Lucas et al., 2009; Sung and Chung, 1997). A few studies have been conducted to determine whether mixtures of PGPR strains can enhance biological control activity of multiple plant diseases by antagonism. In addition to biological control, mixtures of PGPR enhanced the plant growth on germination rate of seeds in petri dishes (Jahanian et al., 2012), shoot and root weight in the greenhouse (Siddiqui and Shaukat, 2002), yield in the field (Nandakumar et al., 2001). Root architecture plays an important role in plant development (Boot, 1989), and most studies have focused on assessing growth promotion by root weight (Khalid et al., 2004). In addition, knowledge about the root architecture induced by mixtures of PGPR strains is scanty.

In a previous study, four individual PGPR strains AP69 (*Bacillus aerophilus*), AP197, AP199, and AP298 (*B. amyloliquefaciens* subsp. *plantarum*), have shown broad-spectrum biocontrol activity in the growth chamber, against two foliar bacterial pathogens includes *Xanthomonas campestris* pv. vesicatoria (Xcv) and *Pseudomonas syringae* pv. tomato (Pst), and one of two soilborne fungal diseases including *Rhizoctonia solani* and *Pythium ultimum*. The objective of this study is to form mixtures of PGPR strains using selected individual PGPR strains and assess

in plants for both biological control of multiple plant diseases and promotion of plant growth and enhanced root architecture.

2. Materials and Methods

2.1. PGPR cultural

Inoculum of PGPR was grown on tryptic soy agar (TSA) at 28 ℃ for 48 h and a single colony was transferred to tryptic soy broth (TSB) and incubated with shaking (150 rpm) at 28 ℃ for 48 h. The bacterial culture was centrifuged at 3500 rpm for 15 min and the pellet was resuspended in sterile distilled water to 10^6 CFU/ml.

2.2. Pathogens and culture conditions

The fungi, *R. solani* (R1) and *P. ultimum* (Py13), were stored in CMA slants at room temperature for long-term storage. *R. solani* were transferred onto PDA plates and grown for 5 days for experimental use. The millet seed inoculum of *P. ultimum* was prepared as described by Howell (2007). The granules of *P. ultimum* inoculum were ground with a coffee grinder then stored in an autoclaved jar at the room temperature. The bacteria of Xcv 95A. 213 and Pst were maintained in TSB supplemented with 20% glycerol at −80 °C and were transferred on TSA plates for experimental use. A single colony was transferred to TSB and incubated with shaking (150 rpm) at 28 ℃ for 48 h. Bacterial cultures were centrifuged at 3500 rpm for 15 min, and the pellet was resuspended in sterile distilled water to 10^7 CFU/ml.

2.3. Greenhouse study

Two separate experiments were conducted in the greenhouse. In experiment-A, a total of six treatments were used: two treatments consisting of single PGPR strains (AP69 or AP199), a treatment consisting of strain mixture (AP69 and AP199), and one positive control (GB03), a non-bacterized but pathogen-challenged disease control, and a non-treated healthy control. The

specific diseases and hosts tested in the experiment-A included bacterial spot of tomato caused by Xcv, bacterial speck of tomato caused by Pst, and damping-off of cucumber caused by *P. ultimum*. In the experiment-B, a total of six treatments were used: two treatments consisting of single PGPR strains (AP197 or AP298), a treatment consisting of strain mixture (AP197 and AP298), and one positive control (GB03), a non-bacterized but pathogen-challenged disease control, and a non-treated healthy control. The specific diseases and hosts tested in the experiment-B included bacterial spot of tomato, bacterial speck of tomato, and damping-off of pepper caused by *R. solani*. The mixture of PGPR strains were prepared by combining equal proportions of each strain prior to application to the seed. Each experiment was arranged in a randomized complete block design (RCBD) with 10 replications per treatment, and repeated twice.

Nine mycelial plugs (7 mm) of *R. solani* from the edge of a pregrown culture or 1 tsp (4.9 ml) of *P. ultimum* inoculum were applied into 1800 cc commercial potting substrate (Sunshine mix, Sun Gro Horticulture, Agawam, Maine). Ten seeds of pepper or cucumber were placed into the 20-cm-round plastic pot (20 cm diameter × 14 cm tall) 24 h after pathogen inoculation. One ml of PGPR suspension (10^6 CFU/ml) was applied to each seed prior to covering with 570 cc potting medium. Plants were maintained in a temperature controlled greenhouse at the Plant Science Research Center at Auburn University, Alabama, U.S.A. Ambient air temperatures in the greenhouse were maintained at 25°C day / 21°C night throughout the year. Watering procedures were carried out routinely by greenhouse personnel with no application of fertilization. The number of seeds emerged at 21 or 7 days and the number of seedlings survived at 28 or 14 days after sowing the seed were recorded for *R. solani* on pepper and *P. ultimum* on

cucumber, respectively. Incidence of pre-emergence damping-off and of post-emergence damping-off was calculated using the formula:

$$\text{Pre} - \text{emergence damping} - \text{off} = \frac{\text{The number of non} - \text{emerged seeds}}{\text{The number of sown seeds}} \times 100\%$$

$$\text{Post} - \text{emergence damping} - \text{off} = \frac{\text{The number of dead seedlings}}{\text{The number of emerged seedlings}} \times 100\%$$

R. solani root lesions were rated using a 0-5 rating scale, where 0=healthy, 1=<10% dark brown lesion at crown, 2= 10-25% of the crown girdled with necrotic lesion, 3= 26-50% of the crown girdled with necrotic lesion, 4= 51-75% of the crown girdled with necrotic lesion, 5= 76-100% of the crown girdled with necrotic lesions. *P. ultimum* root rot severity was rated using a 0-4 rating scale, 0 = healthy white roots at crown with abundant root branching and good overall length, 1 = short and stubby white roots at crown with restricted overall length, 2 = stubby roots at crown with some browning, 3 = severely stunted plant with few roots at crown and browning of main root, 4= nonrecoverable root.

Tomato seeds were raised in germination trays containing 25 cm^3 holes for three weeks, and then transplanted into a 10-cm-square plastic pot filled with 600 cc potting substrate. Two weeks after transplanting, plants were sprayed with PGPR suspension (10^6 CFU/ml) and sterile distilled water through foliar application. PGPR-inoculated plants were placed into a dew chamber (100% humidity) under darkness for two days at 24 ℃, and were transferred to the greenhouse. Three days after spraying with PGPR, plants were challenge-inoculated with Xcv or Pst by spraying the whole plant. Pathogen-inoculated plants were placed into the same dew chamber for two days, then placed in the greenhouse. Plants were watered daily. Disease severity was evaluated at 7 days for Xcv and 14 days for Pst after pathogen challenge. In the rating of disease, four compound leafs were chosen from the bottom of the whole plant, and the disease severity of

compound leafs were averaged by the disease severity of leaflets on that compound leaf. The leaflet was rated using the 0-4 rating scale, where 0=Healthy leaflet, 1= <20% necrotic area of leaflet, 2 = 20-50% necrotic area of leaflet, 3= 51-80% necrotic area of leaflet, 4 = 80-100% necrotic area of leaflet or fully dead leaflet.

Plants were harvested at the same time and shoot fresh weight (SFW), root fresh weight (RFW), and shoot dry weight (SDW) (oven dry at 90 ℃ for 48 h) were measured. Plant roots were analyzed for root morphology using the WinRHIZO Arabidopsis software v2009c 32 bit (Regent Instruments, Quebec, Canada) system connected to an Epson XL 10000 professional scanner. Each root system was evenly spread apart in a water layer on a transparent tray and imaged at a resolution of 400 dpi (dots per inch) (Bauhus and Messier, 1999, Costa, et al., 2000). The following root characteristics were determined: total root length (cm) (TRL), root surface area (cm^2) (RSA), root volume (cm^3) (RV), and total root length of very thin roots with diameter range of 0-0.5mm. Once the root morphological characteristics were determined, root samples were oven dried (90 ℃) to determine root dry weight (RDW).

2.4. Statistical analysis

All data were analyzed by analysis of variance (ANOVA), and the treatment means were separated by using Fisher's protected least significant difference (LSD) test at P=0.05 using SAS 9.4 software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Experiment-A

3.1.1. *X. campestris* pv. vesicatoria

Treatments AP197, AP298, mixture (AP197 + AP298), and positive control (GB03) significantly decreased the disease severity of *X. campestris* pv. vesicatoria ($P < 0.05$), and the

mixture exhibited a higher level of disease reduction compared with either AP197 and AP298 alone (Table 1). Only the mixture significantly increased the root fresh weight, root dry weight, root surface area and total length of fine roots compared with the disease control, and most of the plant growth parameters and root morphology parameters of the mixture were statistically equivalent to the healthy control.

3.1.2. *P. syringae* pv. tomato

Treatments AP197 and the mixture (AP197 + AP298) significantly decreased the disease severity of *P. syringae* pv. tomato ($P < 0.05$) (Table 2). Only the mixture significantly increased all four tested plant growth parameters and 4 root morphology parameters compared with the disease control. Individual PGPR treatments AP197 and AP298 significantly increased 3 out 4 tested plant growth parameters and 4 root morphology parameters compared with the disease control. Most of the plant growth parameters and root morphology parameters of the mixture were higher than the individual PGPR treatments AP197 and AP298, and statistically equivalent to the healthy control.

3.1.3. *R. solani*

Treatments AP197, AP298, mixture (AP197 + AP298), and positive control (GB03) had a significant effect ($P <0.05$) on plant survival, reduction of disease severity, and enhanced 4 plant growth parameters and 4 root morphology parameters compared with the disease control (Table 3). AP298 significantly reduced the pre-emergence damping-off and AP197 significantly reduced the post-emergence damping-off, while the mixture significantly reduced both phases of damping-off. Treatments AP197, mixture (AP197 + AP298), and positive control (GB03) significantly reduced the disease severity of surviving plants. There were no significant differences of plant growth parameters among AP198, AP298, and mixture, however AP197 showed a higher level of root morphology parameters compared with mixture.

3.2. Experiment-B

3.2.1. *X. campestris* pv. vesicatoria

Treatments AP69, AP199, mixture (AP69 + AP199), and positive control (GB03) significantly decreased the disease severity of *X. campestris* pv. vesicatoria ($P < 0.05$) compared to the disease control, and AP69 exhibited a higher level of disease reduction compared with other treatments (Table 4). The mixture and positive control significantly increased the dry mass and 4 root morphology parameters compared to the disease control, while individual treatments AP69 and AP199 increased most of these parameters. Positive control exhibited a higher level of root dry weight, root volume and total length of fine roots compared with mixture.

3.2.2. *P. syringae* pv. tomato

Treatments AP69, AP199 and the mixture (AP69 + AP199) significantly decreased the disease severity of *P. syringae* pv. tomato (Table 5), and increased all the root morphology parameters compared with the disease control ($P <0.05$). Only the mixture significantly increased the root dry weight compared with the disease control.

3.2.3 *P. ultimum*

Treatments AP69 and AP199 had a significant effect ($P < 0.05$) on plant survival. Mixture significantly reduced the pre-emergence damping-off and AP69 significantly reduced the post-emergence damping-off, while AP199 significantly reduced both phases of the damping-off compared to the disease control. All the treatments exhibited the plant protection for the surviving plants. Only AP199 significantly enhanced the shoot dry weigh. AP199 and the mixture significantly increased the root dry weight and 4 root morphology parameters.

4. Discussion

Results presented here demonstrate that individual PGPR treatments exhibited significant antagonistic activity against three pathogens inoculated individually, and promoted the plant growth and root morphology. The mixture of PGPR strains exhibited a general trend toward to greater disease suppression and plant growth promotion.

Overall, in two separate experiments consisted of three different tests repeated twice, our mixture resulted in better levels of disease suppression compared with individual PGPR strains, and the single PGPR strains showed a slightly better level of disease suppression compared with the positive control (GB03) (Table 7 and 8). In the experiment-A, means of disease reduction was 35.1% for the mixture (AP197+298) and 29.1%, 18.4%, and 15.2% for AP197, AP298, and the positive control, respectively. In the experiment-B, means of disease reduction was 26.0% for mixture (AP69+199) and 31.1%, 25.0%, and 19.7% for AP197, AP298, and the positive control, respectively. These results are in agreement with previous studies by Pierson and Weller (1994), Raupach and Kloepper (1998), Jetiyanon and Kloepper (2002), Jetiyanon et al. (2003), Domenech et al. (2006), Lucas et al. (2009), and Zhang et al. (2010), which demonstrated that certain mixtures of PGPR strains were more suppressive to plant diseases than individual PGPR strains. The combination of different mechanisms for pathogen suppression of each individual PGPR strain may explain the success by mixtures of PGPR. In a study of biological control of rice sheath blight, Sung and Chung (1997) found that a combination of four PGPR strains, in which two strains produced chitinase and another two produced antibiotics, resulted in a synergistic effect on the disease suppression. GB03 has been used as a representative commercialized strain for biological control against a broad spectrum of plant pathogens, including *Pythium* spp. (Corrêa et al., 2010), *Rhizoctonia* spp. (Brewer and Larkin, 2005),

Fusarium spp. (Brannen and Kenney, 1997), *Pseudomonas* spp. (Raupach and Kloepper, 1998), *Xanthomonas* spp. (Chandler et al., 2011). In the current study, the comparison to GB03 indicated that some PGPR strains were as effective or better than this commercially strain.

PGPR treatments not only exhibited defense to multiple plant disease, but also promoted plant growth of different hosts in the presence of pathogens with a general trend for best results with mixture (Table 7 and 8). In experiment-A, means of dry mass increase was 28.1% for mixture (AP197+298) and 19.0%, 22.9%, and 12.3 % for AP197, AP298, and positive control, respectively. In the experiment-B, means of dry mass increase was 35.7% for mixture (AP69+199) and 11.8%, 37.3%, and 17.7% for AP197, AP298, and positive control, respectively. Our finding is similar to the results of Goudjal et al. (2014) in a study of tomato disease, in which two isolates of actinomycetes provided biological control of *R. solani* damping-off and promoted the growth of plant shoots and roots. In contrast, a mixture of two bacteria tested in our study, and enhanced plant growth more than the same strains used alone, providing results similar to those obtained by other authors using mixtures of PGPR strains (Jetiyanon et al., 2003; Nandakumar et al., 2001; Pierson and Weller, 1994). Many mechanisms have been related to plant growth elicited by PGPR. First, PGPR can affect plant growth directly by biological nitrogen fixation (Bhattacharjee et al., 2008), solubilization of mineral phosphate (Yazdani et al., 2009), secretion of plant hormones (Idris et al., 2007), and siderophore production (Yu et al., 2011). Second, PGPR can affect plant growth indirectly by preventing the deleterious effects of pathogens. All the strains tested in the current study already exhibited multiple traits related to plant growth promotion and broad-spectrum biocontrol activity *in vitro*. Additive effects may exist between plant growth promotion and biological control: PGPR promotes the growth of the entire plant, which can result in the plant having increased tolerance to disease (El-Tarabily et al.,

2009), and conversely biological control of plant diseases by PGPR may indirectly result in promotion of plant growth (Beneduzi et al., 2012). Future research is needed to gain understanding about the plant growth promotion elicited by PGPR treatments in the absence of pathogens.

In addition to the disease suppression and plant growth promotion, application of PGPR treatments has also been shown to affect plant root morphology with a general trend for best results with a mixture. PGPR treatments increased total root length, root surface area, root volume, and total root length of very thin roots with diameter range of 0-0.5mm (Table 1-6). This finding is consistent with previous works and has already been well demonstrated in several crops including tomato (Gamalero et al., 2002), cucumber (Gamalero et al., 2008), chickpea (Shahzad et al., 2010). It has been demonstrated that root morphology parameters play an important role in plant development due to nutrient uptake is more dependent on root length or root surface area than total root biomass (Boot, 1989). Therefore, an enhancement of nutrient uptake may be detected by PGPR treatments, which has been reported by Gamalero et al. (2004) in which an increase in P acquisition that was related to plant growth was found with PGPR treatments. In the future, nutrient analysis of PGPR treated plants should be performed.

It is also important to note that plant growth and root morphology with a mixture of PGPR treatments in the presence of pathogen, reached a statistically equivalent level of the healthy control in the absence of pathogen (Table 1, Table 2, and Table 5). Interestingly, an increase in root weight and root morphology was seen with foliar application of PGPR. This enhancement of root weight may be caused by the enhancement of photosynthesis which provides the basic energy to the root. Conversely, the enhancement of root can absorb more water and nutrition from the soil to support the shoot growth.

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Table 1. Protection of tomato from bacterial rot caused by *Xanthomonas campestris pv.* vesicatora in greenhouse in the experiment-A.

Strain[ab]	Disease severity[c]	Plant growth parameters				Root morphology parameters[d]			
		Shoot Fresh Weight (g)	Root Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Total length 0<D<0.5 (mm) Fine Roots
Disease	1.88 a	15.48 a	3.01 c	1.38 ab	0.17 c	1054 b	135 c	1.34 b	868 c
AP197	1.44 b	15.16 a	3.35 bc	1.28 b	0.19 bc	1116 b	144 bc	1.49 b	917 bc
AP298	1.53 b	16.99 a	3.40 bc	1.35 b	0.19 bc	1080 b	138 bc	1.41 b	899 c
197+298	1.23 c	17.67 a	3.62 ab	1.41 ab	0.20 b	1200 ab	157 ab	1.58 b	1026 ab
GB03	1.50 b	16.65 a	3.35 bc	1.36 ab	0.18 bc	1159 b	148 bc	1.49 b	958 abc
Healthy	0.00 d	17.26 a	4.16 a	1.61 a	0.24 a	1323 a	176 a	1.88 a	1075 a
$LSD_{0.05}$	0.20	2.64	0.55	0.24	0.03	149	20	0.25	119

[a]The experimental design was a randomized complete block with ten replications per treatment. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]One plant was in each replication. Plants were sprayed with PGPR suspension (10^6 CFU/ml) two weeks after transplanting, and were challenge-inoculated with pathogen solutions (10^7 CFU/ml) three days after inoculating PGPR. Strain AP197, AP298 and GB03=*Bacillus amyloliquefaciens* subsp. *plantarum*.

[c]Harvest and disease severity rating were done at 7 days after posting pathogen challenge. In the rating of disease, four compound leafs were chosen from the bottom of the whole plant, and the disease severity of compound leafs were averaged by the disease severity of leaflets on that compound leaf. The leaflet was rated using the 0-4 rating scale, where 0=Healthy leaflet, 1= <20% necrotic area of leaflet, 2 = 20-50% necrotic area of leaflet, 3= 51-80 % necrotic area of leaflet, 4 = 80-100 % necrotic area of leaflet, or fully dead leaflet.

[d] Plant roots were analyzed for root morphology using the WinRHIZO Arabidopsis software connected to an professional scanner.

Table 2. Protection of tomato from bacterial speck caused by *Pseudomonas syringae pv.* tomato in greenhouse in the experiment-A.

Strain[ab]	Disease severity[c]	Plant growth parameters				Root morphology parameters[d]			
		Shoot Fresh Weight (g)	Root Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Total length 0<D<0.5 (mm) Fine Roots
Disease	1.32 a	27.16 c	8.38 d	2.71 c	0.42 b	2086 c	285 d	3.18 c	1681 c
AP197	0.80 b	28.72 b	8.68 c	2.97 b	0.44 ab	2216 b	312 b	3.51 a	1708 c
AP298	1.13 ab	28.83 b	8.48 c	2.91 b	0.45 ab	2017 c	300 c	3.52 a	1555 d
197+298	0.80 b	30.52 a	9.14 b	3.05 a	0.48 a	2455 a	322 a	3.48 a	1870 b
GB03	1.17 ab	27.39 c	8.38 c	2.84 c	0.42 b	2065 c	291 cd	3.33 b	1583 d
Healthy	0 c	30.43 a	9.49 a	2.81 a	0.44 ab	2445 a	322 a	3.42 ab	1916 a
$LSD_{0.05}$	0.49	0.72	0.33	0.10	0.05	74	9	0.11	39

[a]The experimental design was a randomized complete block with ten replications per treatment. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]One plant was in each replication. Plants were sprayed with PGPR suspension (10^6 CFU/ml) two weeks after transplanting, and were challenge-inoculated with pathogen solutions (10^7 CFU/ml) three days after inoculating PGPR. Strain AP197, AP298 and GB03=*Bacillus amyloliquefaciens* subsp. *plantarum*.

[c]Harvest and disease severity rating were done at 14 days after posting pathogen challenge. In the rating of disease, four compound leafs were chosen from the bottom of the whole plant, and the disease severity of compound leafs were averaged by the disease severity of leaflets on that compound leaf. The leaflet was rated using the 0-4 rating scale, where 0=Healthy leaflet, 1= <20% necrotic area of leaflet, 2 = 20-50% necrotic area of leaflet, 3= 51-80 % necrotic area of leaflet, 4 = 80-100 % necrotic area of leaflet, or fully dead leaflet.

[d] Plant roots were analyzed for root morphology using the WinRHIZO Arabidopsis software connected to an professional scanner.

Table 3. Protection of Pepper from damping-off caused by *Rhizoctonia solani* in growth chambers in greenhouse in the experiment-A.

Strain [ab]	Survive	Disease Incidence		Disease Severity		Plant growth parameters				Root morphology parameters [g]			
		Pre [c]	Post [d]	Standing severity [e]	All severity [f]	Shoot Fresh Weight (g)	Root Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Total length 0<D<0.5 (mm) Fine Roots
Disease	3.50 c	85.0 ab	26.1 a	0.71 a	2.94 a	2.10 d	0.31 d	0.176 d	0.015 d	221 e	25 e	0.224 e	184 e
AP197	4.75 b	79.0 bc	5.8 b	0.31 bc	2.33 cd	3.13 b	0.54 bc	0.250 bc	0.023 bc	426 b	45 b	0.382 b	373 b
AP298	4.70 b	72.0 c	13.9 ab	0.55 ab	2.40 c	3.48 b	0.54 bc	0.270 b	0.024 b	362 c	39 c	0.327 c	319 c
197+298	4.50 b	77.0 c	7.5 b	0.34 b	2.12 d	3.51 b	0.59 b	0.262 b	0.026 b	375 c	40 c	0.360 b	306 c
GB03	4.40 b	86.5 a	13.6 ab	0.37 b	2.66 b	2.68 c	0.49 c	0.231 c	0.020 c	328 d	32 d	0.258 d	266 d
Healthy	8.90 a	7.5 d	0.0 b	0.00 c	0.44 e	7.67 a	1.58 a	0.515 a	0.043 a	625 a	67 a	0.572 a	545 a
$LSD_{0.05}$	0.77	7.4	16.0	0.32	0.25	0.42	0.09	0.029	0.003	34	3	0.027	28

[a]The experimental design was a randomized complete block with ten replications per treatment. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]Ten seeds were sowed in each replication. Pathogen was inoculated 24 hours before sowing the seed, and PGPR was dropped 1.0 ml (10^7 CFU/ml) per seed. Strain AP197, AP298 and GB03=*Bacillus amyloliquefaciens* subsp. *plantarum*.

[c]Pre= Incidence of pre-emergence damping off. Values were determined 7 days after sowing the seed. Pre-emergence damping-off = (The number of non-emerged seeds) / (The number of sown seeds) ×100%.

[d]Post= Incidence of post-emergence damping off. Values were determined 14 days after sowing the seed. Post-emergence damping-off = (The number of dead seedlings) / (The number of emerged seedlings) ×100%.

[e]The standing seedling was rated using the 0-4 rating scale, where 0 = Healthy seedling, 1 = small, light brown lesions on <25% of root, 2 = 25 to 49% of the crown girdled with necrotic lesion, 3 = 50 to 74% of the crown girdled with necrotic lesion, 4 = 75 to 100% of the crown girdled with necrotic lesion or dead.

[f]All the seeds and seedlings were rated using the same 0-4 rating scale.

[g] Plant roots were analyzed for root morphology using the WinRHIZO Arabidopsis software connected to an professional scanner.

Table 4. Protection of tomato from bacterial rot caused by *Xanthomonas campestris pv.* vesicatora in greenhouse in the experiment-B.

Strain[ab]	Disease severity[c]	Plant growth parameters				Root morphology parameters[d]			
		Shoot Fresh Weight (g)	Root Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Total length 0<D<0.5 (mm) Fine Roots
Disease	2.15 a	4.48 bc	0.70 d	0.21 b	0.026 e	225 e	26 d	0.260 d	199 e
AP69	1.57 c	4.76 ab	0.79 c	0.26 a	0.027 de	288 bc	32 bc	0.273 d	256 bc
AP199	1.77 bc	4.98 a	1.08 b	0.26 a	0.030 cd	299 b	31 c	0.270 d	266 b
69+199	1.88 b	4.51 abc	1.08 b	0.27 a	0.031 c	263 d	32 bc	0.292 c	220 d
GB03	1.84 b	3.59 d	1.07 b	0.27 a	0.034 b	272 cd	34 b	0.338 b	249 c
Healthy	0.00 d	4.02 cd	1.25 a	0.26 a	0.048 a	367 a	45 a	0.442 a	321 a
$LSD_{0.05}$	0.26	0.50	0.08	0.02	0.003	19	2	0.018	17

[a]The experimental design was a randomized complete block with ten replications per treatment. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]One plant was in each replication. Plants were sprayed with PGPR suspension (10^6 CFU/ml) two weeks after transplanting, and were challenge-inoculated with pathogen solutions (10^7 CFU/ml) three days after inoculating PGPR. Strain AP197, AP298 and GB03=*Bacillus amyloliquefaciens* subsp. *plantarum*.

[c]Harvest and disease severity rating were done at 7 days after posting pathogen challenge. In the rating of disease, four compound leafs were chosen from the bottom of the whole plant, and the disease severity of compound leafs were averaged by the disease severity of leaflets on that compound leaf. The leaflet was rated using the 0-4 rating scale, where 0=Healthy leaflet, 1= <20% necrotic area of leaflet, 2 = 20-50% necrotic area of leaflet, 3= 51-80 % necrotic area of leaflet, 4 = 80-100 % necrotic area of leaflet, or fully dead leaflet.

[d] Plant roots were analyzed for root morphology using the WinRHIZO Arabidopsis software connected to an professional scanner.

Table 5. Protection of tomato from bacterial speck caused by *Pseudomonas syringae pv.* tomato in greenhouse in the experiment-B.

Strain[ab]	Disease severity[c]	Plant growth parameters				Root morphology parameters[d]			
		Shoot Fresh Weight (g)	Root Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Total length 0<D<0.5 (mm) Fine Roots
Disease	1.53 a	24.17 b	5.67 c	2.59 a	0.339 b	1748 c	236 b	2.47 c	1364 b
AP69	0.60 c	27.77 a	6.48 bc	2.73 a	0.371 ab	1953 ab	264 a	2.77 ab	1576 a
AP199	0.80 bc	26.37 ab	6.72 b	2.71 a	0.386 ab	2023 ab	268 a	2.81 ab	1613 a
69+199	0.62 c	27.61 a	7.71 a	2.77 a	0.409 a	2071 a	268 a	2.92 a	1607 a
GB03	0.88 b	27.40 a	6.87 b	2.72 a	0.386 ab	1909 b	258 a	2.81 ab	1500 ab
Healthy	0.00 d	25.66 ab	6.28 bc	2.56 a	0.372 ab	1973 ab	256 a	2.65 bc	1559 a
$LSD_{0.05}$	0.49	3.07	0.82	0.34	0.049	156	19	0.25	174

[a]The experimental design was a randomized complete block with ten replications per treatment. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]One plant was in each replication. Plants were sprayed with PGPR suspension (10^6 CFU/ml) two weeks after transplanting, and were challenge-inoculated with pathogen solutions (10^7 CFU/ml) three days after inoculating PGPR. Strain AP197, AP298 and GB03=*Bacillus amyloliquefaciens* subsp. *plantarum*

[c]Harvest and disease severity rating were done at 14 days after posting pathogen challenge. In the rating of disease, four compound leafs were chosen from the bottom of the whole plant, and the disease severity of compound leafs were averaged by the disease severity of leaflets on that compound leaf. The leaflet was rated using the 0-4 rating scale, where 0=Healthy leaflet, 1= <20% necrotic area of leaflet, 2 = 20-50% necrotic area of leaflet, 3= 51-80 % necrotic area of leaflet, 4 = 80-100 % necrotic area of leaflet, or fully dead leaflet.

[d] Plant roots were analyzed for root morphology using the WinRHIZO Arabidopsis software connected to an professional scanner.

Table 6. Protection of cucumber from damping-off caused by *Pythium ultimum* in greenhouse in the experiment-B.

Strain [ab]	Survival	Disease Incidence		Disease Severity		Plant growth parameters				Root morphology parameters [g]			
		Pre [c]	Post [d]	Standing severity [e]	All severity [f]	Shoot Fresh Weight (g)	Root Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Total length 0<D<0.5 (mm)
Disease	0.85 c	85.0 ab	38.2 a	2.00 a	3.37 a	2.43 c	0.21 c	0.112 c	0.0065 c	69 d	8 d	0.073 c	60 d
AP69	1.60 b	79.0 bc	22.4 b	1.08 b	3.18 bc	3.12 c	0.36 bc	0.136 c	0.0111 bc	129 cd	15 cd	0.140 bc	111 cd
AP199	1.95 b	72.0 c	22.7 b	0.91 bc	3.05 c	6.27 b	0.63 b	0.276 b	0.0194 b	225 bc	26 bc	0.233 b	193 bc
69+199	1.40 bc	77.0 c	28.6 ab	1.04 b	3.17 bc	5.27 bc	0.63 b	0.245 bc	0.0194 b	286 b	31 b	0.262 b	246 b
GB03	0.80 c	86.5 a	33.6 ab	0.84 bc	3.30 ab	2.98 c	0.27 c	0.140 bc	0.0085 c	100 cd	11 d	0.097 c	86 cd
Healthy	9.25 a	7.5 d	0.0 c	0.50 c	0.30 d	36.47 a	3.22 a	1.450 a	0.0878 a	696 a	75 a	0.664 a	591 a
$LSD_{0.05}$	0.64	7.4	15.3	0.48	0.16	3.04	0.31	0.138	0.0099	143	14	0.122	125

[a]The experimental design was a randomized complete block with ten replications per treatment. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]Ten seeds were sowed in each replication. Pathogen was inoculated 24 hours before sowing the seed, and PGPR was dropped 1.0 ml (10^7 CFU/ml) per seed. Strain AP197, AP298 and GB03=*Bacillus amyloliquefaciens* subsp. *plantarum*.

[c]Pre= Incidence of pre-emergence damping off. Values were determined 7 days after sowing the seed. Pre-emergence damping-off = (The number of non-emerged seeds) / (The number of sown seeds) ×100%.

[d]Post= Incidence of post-emergence damping off. Values were determined 14 days after sowing the seed. Post-emergence damping-off = (The number of dead seedlings) / (The number of emerged seedlings) ×100%.

[e]The standing seedling was rated using the 0-4 rating scale, where 0 = Healthy seedling, 1 = small, light brown lesions on <25% of root, 2 = 25 to 49% of the crown girdled with necrotic lesion, 3 = 50 to 74% of the crown girdled with necrotic lesion, 4 = 75 to 100% of the crown girdled with necrotic lesion or dead.

[f]All the seeds and seedlings were rated using the same 0-4 rating scale.

[g] Plant roots were analyzed for root morphology using the WinRHIZO Arabidopsis software connected to an professional scanner.

Table 7. Summary of suppression of three different pathogens and plant growth promotion by individual plant growth-promoting rhizobacteria (PGPR) strains and mixture of PGPR over two repeated trials in the experiment-A

Treatments[a]	Mean percentage disease reduction				Mean percentage dry mass increase							Mean percentage root morphology parameters increase						
	Xcv[b]	Pst[b]	*R. solani*	**Mean**	Xcv		Pst		*R. solani*		**Mean**	Xcv		Pst		R. solani		**Mean**
					SDW[c]	RDW[c]	SDW[c]	RDW[c]	SDW[c]	RDW[c]		RSA[c]	TLF[c]	RSA[c]	TLF[c]	RSA[c]	TLF[c]	
AP197	23.4	39.4	24.6	**29.1**	-7.2	11.8	9.6	4.8	42.0	53.3	**19.0**	6.7	5.6	9.5	1.6	80.0	102.7	**34.4**
AP298	18.6	14.4	22.3	**18.4**	-2.2	11.8	7.4	7.1	53.4	60.0	**22.9**	2.2	3.6	5.3	-7.5	56.0	73.4	**22.2**
AP197+298	34.6	39.4	31.4	**35.1**	2.2	17.6	12.5	14.3	48.9	73.3	**28.1**	16.3	18.2	13.0	11.2	60.0	66.3	**30.8**
GB03	20.2	11.4	13.9	**15.2**	-1.4	5.9	4.8	0.0	31.3	33.3	**12.3**	9.6	10.4	2.1	-5.8	28.0	44.6	**14.8**
Healthy	100.0	100.0	85.8	**95.3**	16.7	41.2	3.7	4.8	192.6	186.7	**74.3**	30.4	23.8	13.0	14.0	168.0	196.2	**74.2**

[a]Strain AP197, AP298 and GB03=*Bacillus amyloliquefaciens* subsp. *plantarum*.
[b]Xcv= *Xanthomonas campestris pv.* vesicatora, Pst= *Pseudomonas syringe* pv. tomato.
[c]SDW=shoot dry weight (g), RDW=root dry weight (g), RSA= root surface area (cm^2), TLT= total root length of very thin roots with diameter range of 0-0.5mm.

Table 8. Summary of suppression of three different pathogens and plant growth promotion by individual plant growth-promoting rhizobacteria (PGPR) strains and mixture of PGPR over two repeated trials in the experiment-B

Treatments[a]	Mean percentage disease reduction				Mean percentage dry mass increase							Mean percentage root morphology parameters increase						
					Xcv		Pst		*P. ultimum*			Xcv		Pst		*P. ultimum*		
	Xcv[b]	Pst[b]	*P. ultimum*	**Mean**	SDW[c]	RDW[c]	SDW[c]	RDW[c]	SDW[c]	RDW[c]	**Mean**	RSA[c]	TLF[c]	RSA[c]	TLF[c]	RSA[c]	TLF[c]	**Mean**
AP69	27.0	60.8	5.6	**31.1**	23.8	3.8	5.4	9.4	21.4	7.1	**11.8**	23.1	28.6	11.9	15.5	87.5	85.0	**41.9**
AP199	17.7	47.7	9.5	**25.0**	23.8	15.4	4.6	13.9	146.4	19.8	**37.3**	19.2	33.7	13.6	18.3	225.0	221.7	**88.6**
AP69+199	12.6	59.5	5.9	**26.0**	28.6	19.2	6.9	20.6	118.8	19.8	**35.7**	23.1	10.6	13.6	17.8	287.5	310.0	**110.4**
GB03	14.4	42.5	2.1	**19.7**	28.6	30.8	5.0	13.9	25.0	3.1	**17.7**	30.8	25.1	9.3	10.0	37.5	43.3	**26.0**
Healthy	100.0	100.0	91.1	**97.0**	23.8	84.6	-1.2	9.7	1194.6	125.1	**239.5**	73.1	61.3	8.5	14.3	837.5	885.0	**313.3**

[a]Strain AP69= *Bacillus aerophilus*, strain AP199 and GB03=*B. amyloliquefaciens* subsp. *plantarum*.
[b]Xcv= *Xanthomonas campestris pv.* vesicatora, Pst= *Pseudomonas syringe* pv. tomato.
[c]SDW=shoot dry weight (g), RDW=root dry weight (g), RSA= root surface area (cm^2), TLT= total root length of very thin roots with diameter range of 0-0.5mm.

Chapter V Antagonism of black rot (*Xanthomonas campestris* pv. *camperstris*) in cabbage by mixtures of plant growth-promoting rhizobacteria (PGPR) strains

Abstract

Black rot, caused by *Xanthomonas campestris* p*v. campestris*, is the most important and potentially destructive disease of cabbage. The objective of this study was to screen individual PGPR strains and form mixtures with antagonism to control black rot. Twenty-four *Bacillus* spp. strains were tested *in vitro*, and eight strains (AP136, AP201, AP213, AP214 AP218, AP219, AP295 and AP305) were chosen for testing individually in a greenhouse assay. All these strains except AP136 significantly reduced disease in the greenhouse. From these results two mixtures were formed, mixture-1 with the best four individual strains (AP218, AP219, AP295 and AP305), and mixture-2 with the same four strains plus strain AP213 that promoted plant growth in the greenhouse test. These two mixtures and the four individual PGPR strains in mixture 1 were then tested three times in the greenhouse and one time in the field. All the treatments resulted in significant disease suppression. Mixture-2 and strain AP218 caused the highest and most consistent disease reduction in two of the three trials. In addition, mixture-1 showed a positive tendency for growth in the greenhouse test. In the field test, mixture-1, mixture-2 and individual strains AP219 and AP305 significantly reduced disease incidence and head disease severity, as well as increasing yield, compared to the disease control. In conclusion, mixtures of PGPR exhibited stable and consist biocontrol of black rot in cabbage.

1. Introduction

Crucifer crops, including broccoli, cabbage, cauliflower, turnip, and collards, are important vegetable crops and are widely grown in the United States (Williams, 1980). The market value of cabbage has an estimated annual value of over $230 million in the United States (NASS/USDA). Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Xcc), is the most important disease of cabbage and occurs in many crucifer growing regions in the world, including North America, Australia, Europe, Asia, and Africa (Alvarez, 2000; Vicente and Holub, 2013).

Xcc can infect most cruciferous crops at any growth stage and causes significant yield loss when warm, humid conditions follow periods of rainy weather during early crop development (Akhtar, 1989). Although symptoms vary depending on the host, plant age and environmental conditions, the most characteristic symptoms are yellow to yellow-orange "V"-shaped or “U”-shaped chlorotic and necrotic lesions from the margin of the leaf (Akhtar, 1989). Black rot can arise from infected seeds and is spread by splashing rain or sprinkler irrigation. The bacteria enter plants through hydathodes and wounds (Hugouvieux et al., 1998). As the disease progresses, the midrib of the leaf turns black and the vein becomes darkened. The disease becomes systemic in the plant when the pathogen enters the stem. Infected plants are stunted, and heads deteriorate rapidly after harvest (Schaad and Alvarez, 1993). Under warm and wet conditions, a disease epidemic can rapidly occur, causing significant yield losses.

A multitude of disease management approaches have been evaluated against Xcc, each with some success, including cultural, physical, chemical, and biological controls. Culture controls of black rot includes rotating cabbage with plants from other families that are not hosts of black rot, growing plants in fields that have not been in cruciferous crops for at least 2 years, and draining and drying fields (Mew and Natural, 1993). Treating the seed with hot water (50 ℃ for 25 min)

as a physical treatment can significantly reduce bacterial populations on seeds (Nega et al., 2003). Copper-containing fungicides can inhibit this disease (Krauthausen et al., 2011). However, copper resistance to black rot was first identified in a Japanese cabbage cultivar (Early Fuji) (Williams et al., 1972). In addition, many crucifer hybrids with black rot tolerance are available for both fresh and processing commercial production (Kocks and Ruissen, 1996; Williams et al., 1972).

Among all the disease management options for black rot, biological control is attractive given the public concern about the environment. Biological control of plant disease by plant growth-promoting rhizobacteria (PGPR) is a promising strategy for plant protection (Kloepper et al., 1999). Management of black rot on cruciferous crops by antagonistic PGPR strains has been demonstrated in several studies (Massomo et al., 2004; Mishra and Arora, 2012a; Mishra and Arora, 2012b; Monteiro et al., 2005; Wulff et al., 2002). These studies reported the use of a single PGPR strain against black rot. *Pseudomonas fluorescens* strain To7 produced 2, 4-diacetylphloroglucinol managed black rot in cabbage(Mishra and Arora, 2012b), and *Bacillus subtilis* strain BB inhibited three strains of Xcc in four *Brassica* crops (cabbage, cauliflower, rape and broccoli) on different types of soil (Wulff et al., 2002). Although these individual PGPR strains showed biological control activity, several studies have shown that mixtures of PGPR strains can be more effective due to synergistic modes of action (Jetiyanon and Kloepper, 2002; Raupach and Kloepper, 1998). Mixtures of several strains may result in a more stable rhizosphere community, provide several mechanisms of control, and lead to broad-spectrum biocontrol activity on different hosts under diverse field conditions (Domenech et al., 2006; Jetiyanon et al., 2003).

Currently, there is very limited knowledge regarding the biological suppression of black rot in cabbage by the application of mixtures of PGPR strains. The aim of this study was to selected individual PGPR strains for suppressing black rot on cabbage through antagonistic activity, and to investigate whether mixtures of PGPR strains could improve the consistency and level of disease reduction and plant growth in the greenhouse and field.

2. Materials and methods

2.1. PGPR strains and inoculum preparation

In this research, twenty-four PGPR strains were obtained from the PGPR lab in the Department of Entomology and Plant Pathology, Auburn University, and tested in this experiment. The bacteria were maintained in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA), supplemented with 20% glycerol at −80 °C. For *in vitro* tests, inoculum of PGPR was grown on tryptic soy agar (TSA) at 28 ℃ for 48 h. For *in planta* tests, theses strains were used as spore preparations.

2.2. *X. campestris* pv. *campestris* inoculum preparation

The Xcc strain 92 B.243 was grown on Yeast Dextrose Calcium Carbonate Agar plate (YDC) at 28 ℃ for 72 h (Schaad et al., 1988). A single colony was transferred to tryptic soy broth (TSB) and incubated with shaking (150 rpm) at 28 ℃ for 48 h. Bacterial cultures were centrifuged at 3500 rpm for 15 min, and the pellet was resuspended in sterilized water. The concentration was adjusted to 10^8 CFU/ml.

2.3. PGPR antagonistic activity to *X. campestris* pv. *campestris*

An antibiosis technique was developed in which different types of agar were used for the PGPR and the challenged pathogen. Three holes of 13 mm diameter were made into water agar in 85 petri plates, and these were filled with melted tryptic soy agar (TSA). After the TSA

became solid, 10 µl of the PGPR suspension was applied to the TSA disc. Plates were incubated at 28 °C for 48 h, and then exposed to ultraviolet light (1000 x100 µJ /cm2) for 2 min to prevent bacterial growth out of the TSA disc. One ml of Xcc suspension was mixed with 50 ml soft agar (0.4% Agar in 50% TSB) and cooled to 37 ℃. After gently swirling, 7 ml of the resulting suspension was added to each plate. Each plate contained two PGPR strains and a water control placed at approximately equal distances from each other at the edge of the plate. Plates were incubated for two days at 28 ℃. The inhibition zone was measured from the edge of the PGPR strain to the pathogen. Each treatment was repeated three times.

2.4. Preliminary screen in the greenhouse

Kaboko Hybrid Organic Chinese Cabbage Seeds (Park Seed, USA) which is susceptible to Xcc were used. Cabbage seeds were raised in germination trays containing 25 cm^3 holes for two weeks, and then transplanted into 4.5-inch round pot filled with organic potting mix. Two weeks after transplanting, plants were sprayed with PGPR suspension (10^8 CFU/ml). PGPR-inoculated plants were placed into a dew chamber (100% humidity) under darkness for two days at 24 ℃, and were transferred to the greenhouse. Three days after spraying with PGPR, plants were challenge-inoculated with Xcc by spraying the whole plant. Pathogen-inoculated plants were placed into the same dew chamber for two days, then placed in the greenhouse. Plants were watered daily.

Fourteen days after pathogen challenge, total lesion number (TLN) was recorded for each plant. Five leaves from the bottom of each plant were chosen for evaluating the disease index (Figure 1). The disease index for black rot was scored according to a 0~5rating scale, where 0 = leaves which appeared healthy with no symptoms, 1 = slightly chlorotic at the margins of leaves, 2 = chlorotic at margins - chlorotic blotches at the margin of leaves, 3 = chlorosis progressed

toward the midrib of the leaf, 4 = leaves showing extreme chlorotic progression with V-shaped blotches with some one-sided growth and with rapid lower leaf droop, and 5 = brown leaf with some extreme blackening on the vein (Fig. 1). Plants were harvested at the same time and the following plant parameters were measured: plant diameter, shoot fresh weight (SFW), shoot dry weight (SDW) (oven dry at 90 ℃), root fresh weight (RFW), and root dry weight (RDW) (oven dry at 90 ℃).

The experimental design was a randomized complete block design with ten treatments and eight replications in each treatment. Treatments included eight PGPR strains (AP136, AP201, AP213, AP214, AP218, AP219, AP295, and AP305), and two controls (a nonbacterized but pathogen-challenged disease control, and a nontreated healthy control). The experiment was repeated once.

2.5. Advanced test of selected individual stains and mixtures in the greenhouse and field

Four individual PGPR strains (AP218, AP219, AP295, and AP305) selected in the preliminary greenhouse screen were tested again. These four strains had shown the best antagonistic capacity and were mixed together as the mixture-1, and for mixture-2, one strain (AP213) that showed growth promotion was added. A total of seven treatments were used: four treatments consisting of the single PGPR strains (AP218, AP219, AP295 and AP305); two treatments consisting of strain mixtures (Mixture-1 and Mixture-2), and one control (a nonbacterized but pathogen-challenged disease). Methods were the same as previously described, and the biocontrol capacity was quantified by the total lesion number. The mixture of PGPR strains were prepared by combining equal proportions of each strain prior to application to the seed. The experiment was conducted three times.

The same treatments were tested once at E.V. Smith Research Center, Shorter, AL, USA (32.45 N, 85.88 W). The soil type is sandy loam. The total amount of rainfall during this experiment was 116 mm. The maximum average temperature ranged from 17.17 ℃ to 24.44 ℃, while the minimum varied form 2.11 ℃ to 10.17 ℃.

Seedlings were transferred into the field after growing for six weeks in the greenhouse. Two weeks after transplanting, PGPR suspensions (10^8 CFU/ml) were sprayed on the whole plant. Three days after inoculation with the PGPR, the whole plant was sprayed with the pathogen (10^8 CFU/ml). The inoculations of PGPR and pathogen were done in the late afternoon to prevent rapid drying. This experiment was laid out in a randomized complete block design, and each treatment had six plots. Each plot contained 10 plants (2 rows of 5 plants each), and the distance between rows was 60 cm and within rows was 45cm.

The biological control effect was quantified by recording the incidence three and ten weeks after transplanting, and assessing the external, head surface, and internal black rot symptoms 71 days after transplanting.

For scoring the external black rot index (EBR index), leaves not forming part of the head were examined for black rot symptoms and assessed as follows: EBR index =(0a + 1b + 2c + 3d + 4e)/T, where: 0, 1, 2, 3 and 4 indicate, respectively, none, >0–10%, 11–20%, 21–30% and >30% of the surface of a leaf showing black rot symptoms; a–e correspond to the number of leaves in the infection category; T is the total number of external leaves.

For recording the head index (HBR index), the surface of the whole head was checked as follows: HBR index = (0a + 1b + 2c + 3d + 4e)/T, where a=No symptom on the head surface; b=the symptoms only on the surface; c= symptoms on the surface and inside of leaf; d=

symptoms on the surface and inside of leaf, and form V-shape lesion; e= symptoms on the surface and inside of leaf, form V-shape lesion and veil discoloration.

For assessing the internal black rot index (IBR index), cabbage heads were cut perpendicularly into quarters and the internal symptoms were assessed as follows: 0 =No discolouration, no symptoms on the heart leaves (healthy plants); 1 = vein discolouration extends <1/2 of the stem, no symptoms on the heart leaves; 2 = vein discolouration extends >1/2 of the stem, no symptoms on the heart leaves; 3 = vein discolouration of stem and 1–3 of the heart leaves and 4 = vein discolouration of stem and on more than 3 heart leaves.

To determine the yield, the whole plant was recorded as the whole yield. After peeling off the leaves that did not form the head part, the yield was recorded as the head yield. The marketable yield was the head after removing any outer leaves with symptoms.

2.6. Statistical analysis

All data were analyzed by analysis of variance (ANOVA), and the treatment means were separated by using Fisher's protected least significant difference (LSD) test at P=0.05 using SAS 9.4 software (SAS Institute, Cary, NC, USA).

3. Results

3.1. PGPR antagonistic activity to *X. campestris* pv. *campestris*

Among twenty-four tested stains, nineteen strains produced inhibition zones and twelve strains produced inhibition zones larger than 10 mm (Table 1). Depending on the isolation source and species of these antagonistic strains, eight strains (AP136, AP201, AP213, AP214, AP218, AP219, AP295, and AP305) were selected for antibiosis tests in the greenhouse (Fig. 1).

3.2. Preliminary screen in the greenhouse

Four PGPR strains (AP218, AP219, AP295, and AP305) showed the best disease reduction of total lesion number and disease severity (Table 2). Strains AP201, AP213, AP214, AP218, AP219, AP295, and AP305 caused significant reduction of lesion numbers, and strains AP201, AP218, AP219, AP295, and AP305 caused > 40% disease reduction compared with the disease control. Strains AP136, AP201, AP213, AP214, AP218, AP219, AP295, and AP305 reduced the disease severity, and treatments AP213, AP218, AP219, AP295, and AP305 exhibited > 35% disease suppression.

Two strains, AP213 and AP295, exhibited better plant growth than other strains or the healthy control (Table 2). Treatment AP213 significantly increased the shoot fresh weight, and treatments AP213 and AP295 increased the diameter of the head cabbage. Meanwhile, the shoot fresh weight, shoot dry weight and plant diameter with treatment AP213 was numerically greater than the healthy control, and the shoot dry weight and diameter with AP295 were also numerically greater than the healthy control.

3.3. Advanced tests in the greenhouse and field

All the PGPR treatments significantly reduced lesion numbers compared to the disease control (Table 3). Individual strain AP218 and mixture-2 showed a higher level of biocontrol capacity than other treatments, reaching to 29.4% and 31.0% disease reduction respectively. In addition, these two treatments were consistent with disease reduction in two of three repeated experiments. Mixture-1 significantly increased the root dry weight and the diameter of cabbage compared to the control (Table 4).

Three weeks after transplanting, all the PGPR treatments significantly delayed pathogen infection (Table 5). Ten weeks after transplanting, treatments AP218 and mixture-1 significantly

inhibited disease incidence. Although the biocontrol effect of all the treatments was not found to be significant in cabbage leaves (EBR index), all the treatments significantly reduced black rot symptoms on the head (HBR index) compared with the control. Internal black rot symptoms (IBR Index) were not observed inside of the cabbage head at harvest time.

Although there are no statistically significant effects on the yield, weight gains with PGPR treatments were consistent (Table 6). In particular, mixture-2 numerically increased the whole yield, head yield and marketable yield by 12.2%, 13.4% and 10.6% respectively. Meanwhile, the whole yield of all the treatments, except treatment AP218, was greater than the disease control, the head yield of all the treatments was bigger than disease control, and the marketable yield of treatment of AP219 and AP305, mixture-1 and mixture-2 was bigger than disease control.

4. Discussion

The results presented here confirmed that individual PGPR strains and mixtures significantly reduced black rot lesion numbers under greenhouse conditions. However, one formed mixture was more consistent and effective against black rot of cabbage, and the same mixture exhibited the best positive tendency on yield growth compared with other treatments in the field.

To efficiently select individual PGPR strains and designate strain mixtures for management of black rot, the screening strategy involved, first, selecting for inhibition of pathogen growth *in vitro* by 24 individual PGPR strains then disease reduction and growth promotion *in planta* by 8 individual PGPR strains. The agar plate test for determining antagonistic potential provides a rapid and easy assay at the initial screening. However, some studies have demonstrated that inherent limitations exist for the *in vitro* assay due to no relation between the size of inhibition zone and disease control in plants (Tolba and Soliman, 2013). Thus, five strains exhibiting an inhibition zone > 10 mm and three strains with an inhibition zone < 10 mm were chosen for

preliminary screening *in planta*. Strains exhibiting the largest inhibition zone were not the best ones for disease reduction in the greenhouse. For example, stain AP 136 had the largest inhibition zone but did not significantly reduce the lesion number *in planta* (Table 1 and Table 2). Raupach and Klopper (1998) proposed the strategies for forming mixtures of biocontrol agents including mixtures of organisms with differential plant colonization patterns; mixtures of antagonists that control different pathogens; mixtures of antagonists with different mechanisms of disease suppression; mixtures of taxonomically different organisms; or mixtures of antagonists with different optimum temperature, pH, or moisture conditions for plant colonization. In some typical studies of mixtures of PGPR, all the possible ways of formulating mixtures were tested and the number of individual strains was less than 4 (Raupach and Kloepper, 1998; Zhang et al., 2010). Mishra and Arora (2012a) combined *Pseudomonas* KA19 and *Bacillus* SE that both were effective against Xcc, and KA19 was better than SE in biological control while SE was better than KA19 in plant growth promotion. In the current study, mixture-1 was formulated by four PGPR strains that showed the best antagonistic capacity in the preliminary screening, and mixture-2 included strains that promoted growth under the disease suppression and all the strains in the mixture-1.

Compatible mixtures of PGPR can give consistent performance and a greater protection than do individual PGPR strains. Jeiyanon et al (2003) tested individual PGPR and mixtures in Thailand during the rainy season and winter season and showed that mixtures more consistently suppressed both disease severity and disease incidence in both seasons than did individual strains. In our studies of three repeated trials in the greenhouse (Table 3), mixture-2 and individual strain AP 218 significantly reduced disease incidence in the first and second trial, while no significant differences were noted among treatments in the third trial. Other treatments, i.e., mixture-1,

AP219 and AP305 showed a significant reduction of lesion numbers once among three repeated trials. Based on combined data for all three trials in the greenhouse, mixture-2 caused a 31% disease reduction, while individual PGPR strains AP218, AP219, AP295, and AP305 caused a 29.4%, 23.0%, 15.0%, 23.4% disease reduction respectively. These results are in agreement with the study by Zhang et al. (2010), in which mixture of INR7+T4+SE56 and INR7+IN937a+T4+SE56 tended to cause higher levels of disease reduction of Phytophthora blight on squash compared to individual PGPR strains. Combination of plant defense mechanisms may enhance plant protection. A wide variety of PGPR metabolites include antibiotic, siderophore, and cell wall degrading enzymes are involved in antagonism (Fernando et al., 2006; Jha and Subramanian, 2014; Sayyed et al., 2013). Among these metabolites, antibiotics were extensively studied (Govindasamy et al., 2011). Some antibiotics have been found to be produced by *Bacillus* spp: zwittermicin A and kanosamine by *B. cereus* strain UW85 biocontrol of damping-off (*Phytophthora medicaginis*) (Stabb et al., 1994), fengycin by *B. subtilis* strain F-29-3 biocontrol of *Rhizoctonia* disease (Vanittanakom et al., 1986), iturin A by *B. amyloliquefaciens* strain B94 biocontrol of *R. solani* (Yu et al., 2002). In the future, those antibiotics that are involved in biocontrol of black rot should be tested.

Foliar application of PGPR could protect plants foliar diseases and increase growth. Capacity to increase plant growth was related to disease protection capacity against black rot. In the greenhouse study, the growth parameters were increased by mixtures of PGPR when the lesion number was reduced (Table 3, and 4). In the field trial, every time that the head black rot index was reduced, increase of yield was observed (Table 5 and 6). These results are in agreement with study by Zhang et al (2004), in which PGPR strains induced disease protection and plant growth promotion. The application method of PGPR is different between our studies through foliar

application and published report through seed treatments and root drenches. It is still unclear about how PGPR exhibit both biocontrol and growth promotion at the same time. This may be answered by the following reasons, 1) some single PGPR strains exhibited both biocontrol activity and production of multiple traits related to plant growth promotion (Ahmad et al., 2008; Wahyudi and Astuti, 2011), and 2) PGPR promote the growth of the entire plant, which can result in the plant having increased tolerance to disease, and conversely biological control of plant diseases by PGPR may indirectly result in promotion of plant growth (Beneduzi et al., 2012). Typically, application of PGPR to the seed or root increased the root weight (Abbasi et al., 2011; Bal et al., 2013). This was caused by improving nutrient release (Adesemoye et al., 2009) and mineral uptake (Selvakumar et al., 2009), producing plant hormone (Etesami et al., 2009) or controlling pathogens in the soil (Liu et al., 2009). In the current study, an increase in the root dry weight exhibited when foliar application of PGPR. This enhancement of root weight may be caused by the enhancement of photosynthesis provide the basic energy to the root. Conversely, the enhancement of root can absorb more water and nutrition from the soil to support the shoot growth.

Treatments that best reduced disease incidence or disease severity were not always the same as those that best enhanced plant growth or yield. In the greenhouse study, mixture-2 supposed the best for growth promotion while mixture-1 exhibited the best for growth promotion. However, mixture-2 reduced the most black rot on the head exhibited the highest yield compared with other treatments in the field. These results are in agreement with the study by Jetiyanon et al (2003), in which the mixture of IN937a +IN937b promoted the highest cucumber fruit weight did not the best for against *Cucumber mosaic virus*. In the future, using different carriers such as peat and talc may be a good way to formulation a stable mixtures of PGPR.

At harvest time, some treatments could inhibit the infection of pathogen (disease incidence) and prevent the spreading of the pathogen in the leaf (disease severity), while some treatments could only prevent the spreading of the pathogen and increase the yield (Table 5 and 6). This indicates that different biochemical or physiological changes occurring within different PGPR-treated cabbage plants. Marketable yield was the head after removing any outer leaves with symptoms may be the best way to evaluate biocontrol effects of black rot on cabbage.

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Table 1. *In vitro* antagonistic activity between individual plant growth-promoting rhizobacteria (PGPR) and *Xanthomonas campsetris* pv. *campestris* 92B. 243.

Treatment	ID	Inhibition zone (mm) [a]
AP7	*Bacillus safensis*	0.00 f
AP18	*Bacillus altitudinis*	0.00 f
AP136	*Bacillus amyloliquefaciens*	11.67 a
AP188	*Bacillus amyloliquefaciens*	1.00 f
AP194	*Bacillus methylotrophicus*	9.00 cd
AP195	*Bacillus methylotrophicus*	11.00 ab
AP197	*Bacillus methylotrophicus*	7.33 ed
AP199	*Bacillus methylotrophicus*	11.33 ab
AP200	*Bacillus methylotrophicus*	11.33 ab
AP201	*Bacillus methylotrophicus*	10.67 abc
AP203	*Bacillus methylotrophicus*	10.00 abc
AP208	*Bacillus methylotrophicus*	10.67 abc
AP209	*Bacillus mojavensis*	0.00 f
AP210	*Bacillus methylotrophicus*	11.00 ab
AP211	*Bacillus methylotrophicus*	9.00 cd
AP212	*Bacillus methylotrophicus*	10.00 abc
AP213	*Bacillus amyloliquefaciens*	10.67 abc
AP214	*Bacillus amyloliquefaciens*	9.67 bc
AP217	*Bacillus solisalsi*	0.00 f
AP218	*Bacillus methylotrophicus*	10.67 abc
AP219	*Bacillus methylotrophicus*	6.00 e
AP282	*Lysinibacillus boronitolerans*	0.00 f
AP295	*Bacillus amyloliquefaciens*	9.00 cd
AP305	*Bacillus amyloliquefaciens*	10.67 abc
Control		0.00 f
$LSD_{0.05}$		1.87

a. The inhibition zone was measured from the edge of PGPR strain to the pathogen. Numbers with different letters show significant difference at $P< 0.05$.

Table 2. Results of the preliminary screening for effects of individual plant growth-promoting rhizobacteria (PGPR) strains on biocontrol capacity and plant growth parameters.

Treatment[ab]	Total lesion number	Disease severity	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weigh (g)	Plant diameter (cm)
Disease Control	4.19 a	3.48 a	14.94 bc	0.82 abcd	0.32 bcd	0.01	23.50 de
AP136	3.6 ab	2.48bc	12.63 c	0.59 d	0.33 bcd	0.02	21.63 e
AP201	2.25c	2.68b	14.33 c	0.65 cd	0.32 bcd	0.01	23.88 ed
AP213	2.94 bc	2.18bcd	25.38 a	1.07 a	0.50 ab	0.01	29.35 a
AP214	3.00 bc	2.50 bc	12.40 c	0.59 d	0.17 d	0.01	23.99 cde
AP218	2.06 c	2.13 bcd	17.44 bc	0.84 abcd	0.32 bcd	0.01	25.89 bcd
AP219	2.31 c	2.08 cd	14.12 c	0.76 bcd	0.25 cd	0.01	24.66 bcde
AP295	2.38c	2.25bcd	20.26 ab	1.03 a	0.50 ab	0.01	27.58 ab
AP305	1.94 c	1.80d	17.37 bc	0.91 abc	0.43 abc	0.01	25.46 bd
Healthy Control	0.00e	0.00 e	24.62 a	1.01 ab	0.57 a	0.03	27.03 abc
$LSD_{0.05}$	1.01	0.60	5.63	0.27	0.21	0.01	3.08

[a]The experiment was arranged by randomized completely block design (RCBD), using 4.5-inch round pots with single cabbage per pot for eight replications per treatment. The experiment was repeated once. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]PGPR strains were spayed to the whole plant. Strain AP136, AP213, AP214 and AP295= *B. amyloliquefaciens*, AP305= *B. amyloliquefaciens* subsp. *plantarum*, AP201, AP218 and AP219=*B. methylotrophicus*. A nonbacterized but pathogen-challenged treatment was used as the control, and a nontreated treatment was used as healthy control.

Table 3. Effects of selected individual plant growth-promoting rhizobacteria (PGPR) strains and mixtures of PGPR strains on cabbage black rot caused by *Xanthomonas campsetris* pv. *campestris* 92B. 243.

Treatment[a]	Number of lesions				% disease reduction
	Trial 1	Trial 2	Trial 3	Mean of 3 trials	
Disease Control	13.58 a	10.22 a	10.08 a	11.03 a	
AP218	8.75 bc	7.20 bcd	7.98 a	7.94 c	29.4
AP219	7.92 c	8.57 ab	9.60 a	8.85 bc	23.0
AP295	11.25 ab	8.20 abc	9.33 a	9.49 b	15.0
AP305	10.58 abc	5.91 d	9.44 a	8.67 bc	23.4
Mixture-1	9.58 bc	9.40 ab	8.58 a	9.09 bc	18.6
Mixrure-2	8.42 bc	6.14 cd	8.80 a	7.89 c	31.0
$LSD_{0.05}$	3.10	2.26	2.65	1.54	

[a]PGPR strains applied as foliar spray. Mixture-1: AP218, AP219, AP295 and AP305; Mixture2: AP213, AP218, AP219, AP295 and AP305.

Table 4. Effects of selected individual plant growth-promoting rhizobacteria (PGPR) strains and mixtures of PGPR strains on plant growth parameters of cabbage.

Treatment[a]	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Plant diameter (cm)
Disease Control	40.46 a	3.17 bc	6.78 a	0.49 b	32.94 c
AP218	35.87 a	3.01 bc	7.86 a	0.52 b	33.76 abc
AP219	38.00 a	3.06 bc	7.17 a	0.48 b	32.53 c
AP295	35.55 a	2.80 c	7.39 a	0.46 b	34.21 abc
AP305	39.06 a	3.26 bc	7.86 a	0.49 b	35.93 a
Mixture-1	40.95 a	3.78 a	7.76 a	0.63 a	35.21 ab
Mixrure-2	41.73 a	3.41 ab	7.78 a	0.54 ab	33.76 bc
$LSD_{0.05}$	6.20	0.49	1.51	0.10	2.43

[a]PGPR strains applied as foliar spray. Mixture-1: AP218, AP219, AP295 and AP305; Mixture2: AP213, AP218, AP219, AP295 and AP305.

Table 5. Effects of individual plant growth-promoting rhizobacteria (PGPR) strains and strain mixtures on incidence and severity of black rot disease in the field.

Treatment[a]	Disease incidence		Disease severity		
	3 weeks after transplanting	10 weeks after transplanting	External black rot index	Head black rot index	Internal black rot index
Disease Control	45.0 a	55.0 ab	1.56 ab	2.17 a	0
AP218	13.3 bc	35.0 d	1.47 b	1.44 b	0
AP219	13.3 bc	46.7 bc	1.57 ab	1.42 b	0
AP295	15.0 b	50.0 abc	1.77 a	1.28 b	0
AP305	8.3 cd	50.0 abc	1.61 ab	1.25 b	0
Mixture-1	16.7 b	41.7 cd	1.53 ab	1.36 b	0
Mixture-2	13.3 bc	60.0 a	1.46 b	1.14 b	0
$LSD_{0.05}$	5.2	10.7	0.23	0.48	0

[a]The experiment was laid out in a complete randomized block design, and each plot contained 10 plants (2 rows of 5 plants each). The experiment was conducted once. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

Table 6. Effects of individual plant growth-promoting rhizobacteria (PGPR) strains and strain mixtures on the yield in the field.

Treatmenta	Whole yield[a]		Head yield[a]		Marketable yield[a]	
	kg per plant	% Increase	kg per plant	% Increase	kg per plant	% Increase
Disease Control	1.54 a		1.14 a		0.91 a	
AP218	1.53 a	-0.2%	1.14 a	0.0%	0.90 a	-1.1%
AP219	1.68 a	9.3%	1.26 a	10.4%	0.96 a	5.8%
AP295	1.59 a	3.3%	1.17 a	2.9%	0.90 a	-0.6%
AP305	1.70 a	10.3%	1.25 a	10.0%	0.99 a	8.5%
Mixture-1	1.62 a	5.1%	1.20 a	5.7%	0.95 a	4.9%
Mixture-2	1.72 a	12.2%	1.29 a	13.4%	1.01 a	10.6%
LSD0.05	0.28		0.23		0.19	

[a] To determine the yield, the whole plant was recorded as the whole yield. After peeling off the leaves that did not form the head part, the yield was recorded as the head yield. The marketable yield was the head after removing any outer leaves with symptoms.

Figure 1. Disease severity of black rot.

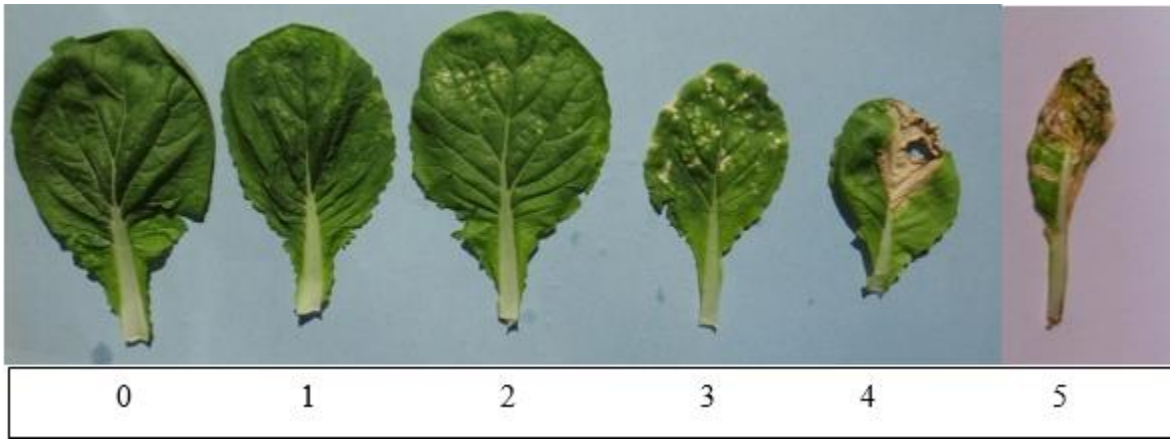
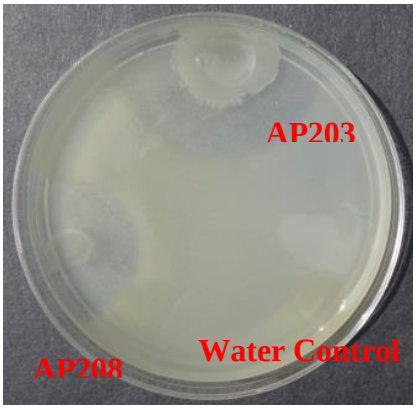
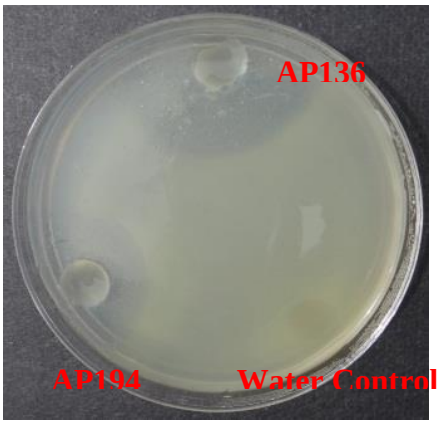


Figure 2. Antagonistic interaction between individual plant growth-promoting rhizobacteria (PGPR) strains and *Xanthomonas campsetris* pv. *campestris* 92B. 243 *in vitro*.





Chapter VI Induction of Systemic Resistance in Chinese cabbage Against Black Rot by Plant Growth-Promoting Rhizobacteria

Abstract

Black rot, caused by *Xanthomonas campestris* p*v. campestris*, is the most important and potentially destructive disease of cabbage. The objective of this study was to select plant growth-promoting rhizobacteria (PGPR) strains and to form strain mixtures with the capacity to elicit induced systemic resistance in Chinese cabbage. Components of this study included testing individual PGPR strains in greenhouse assays, and testing mixtures of PGPR in greenhouse and field tests. In greenhouse tests, 10 of 12 tested individual PGPR strains reduced the number of foliar lesions, and five individual PGPR strains increased fresh shoot weight, dry shoot weight and fresh root weight. Four individual stains (AP136, AP209, AP282 and AP305) were combined in mixture-1, and mixture-2 added three additional stains (AP7, AP18 and AP218) to mix-1. Both mixtures and three individual strains significantly reduced black rot, and mixture-2 increased dry shoot weight and dry root weight. In the field test, all the tested treatments significantly reduced disease incidence at three weeks after transplanting, head disease severity at harvest time, and increased marketable yield compared to the nonbacterized control.

1. Introduction

Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Xcc), is the most important and potentially destructive disease of cabbage (Vicente and Holub, 2013; Williams, 1980). Black rot may arise from systemic infection (infected seeds) and from secondary spread. Infected seed is a source of secondary infections if the bacteria are exuded from the hydathodes, which are natural openings on the leaf edge that connect to the xylem (Akhtar, 1989). Splashing rain or sprinkler irrigation can spread the pathogen from the source plant to hydathodes of neighboring plants (Hugouvieux et al., 1998).

Tactics for management of black rot include using certified, disease-free transplants and seeds, resistant cultivars, heat treatment, biological control, and chemical control (Mew and Natural, 1993; Nega et al., 2003). Biological control can reduce pesticide useage, making it an attractive alternative management option for crop protection (Chandler et al., 2011; Waard et al., 1993). The use of plant growth-promoting rhizobacteria (PGPR) as biopesticides is reported to be an effective way to reduce the use of agrichemicals (Banerjee et al., 2005; Chandler et al., 2011; Subba Rao, 1993). PGPR are beneficial bacteria that influence the growth, yield and nutrient uptake of the plant, and provide biological control of plant disease (Chithrashree et al., 2011; Kloepper and Schroth, 1978; Mia et al., 2010).

PGPR exhibit two major mechanisms of biological control, including direct mode-antagonism and indirect mode-induced systemic resistance (Compant et al., 2005; Kloepper et al., 2004). Biological control of black rot by antagonistic bacteria has been demonstrated experimentally with bacteria to control black rot on crucifers (Massomo et al., 2004; Mishra and Arora, 2012a; Mishra and Arora, 2012b; Monteiro et al., 2005; Wulff et al., 2002). Compared with antagonism, the physiological and metabolic response of the host plant is altered after the induction of ISR,

leading to an enhanced synthesis of plant defense chemicals to challenge the pathogen (Benhamou et al., 1996; Dao et al., 2011). Meanwhile, some PGPR strains have induced systemic resistance against multiple plant diseases (Kloepper et al., 1997; Ramamoorthy et al., 2001).In addition to having the capacity for biocontrol, PGPR has been reported to enhance plant growth directly by a wide variety of mechanisms: biological nitrogen fixation, solubilization of mineral phosphate, secretion of plant hormones, and siderophore production (Antoun and Prevost, 2006; Lugtenberg and Kamilova, 2009; Vessey, 2003).

Although the beneficial effects of PGPR on plants usually are separated into two categories: biological control and growth promotion, there is a close relationship between them (Mariano and Kloepper, 2000). A single PGPR strain can exhibit both of these effects through multiple mechanisms (Wahyudi and Astuti, 2011). In search of efficient PGPR strains, multiple traits related to plant growth promotion (PGP) and biocontrol activity were tested together during the screening process, and selected strains showed multiple functions related to crop production (Ahmad et al., 2008; Praveen Kumar et al., 2014; Wahyudi and Astuti, 2011).

Currently there is very limited knowledge available regarding the biological suppression of black rot in cabbage by induced systemic resistance. Objectives of this study were to 1) test individual strains for multiple traits related to PGP *in vitro* and induction of systemic resistance to black rot in cabbages *in planta*, and 2) form mixtures of PGPR strains based on objective 1 and evaluate the select individual strains and mixtures in the greenhouse and field.

2. Materials and methods

2.1. PGPR strains and inoculum preparation

Twelve selected PGPR strains were used: *Bacillus safensis* strain AP7, *B. altitudinis* strain AP18, *B. mojavensis* strain AP209, *B. solisalsi* strain AP217, *Lysinibacillus boronitolerans*

strain AP282, *B. amyloliquefaciens* strains AP136, AP188, AP213, and AP295, *B. amyloliquefaciens* subsp. *plantarum* strain AP305, *B. methylotrophicus* strains AP218 and AP219. The bacteria were maintained in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA), supplemented with 20% glycerol at −80 °C. For *in vitro* tests, inoculum of PGPR was grown on tryptic soy agar (TSA) at 28 ℃ for 48 h. For *in planta* tests, theses strains were used as spore preparations.

2.2. *X. campestris pv. campestris*(Xcc) inoculum preparation

The Xcc strain 92B. 243 was grown on Yeast Dextrose Calcium Carbonate Agar plate (YDC) at 28 ℃ for 72 h (Schaad et al., 1988). A single colony was transferred to tryptic soy broth (TSB) and incubated with shaking (150 rpm) at 28 ℃ for 48 h. Bacterial cultures were centrifuged at 3500 rpm for 15 min, and the pellet was resuspended in sterilized water. The concentration was adjusted to 10^8 CFU/ml.

2.3. Preliminary screening

Four traits related to plant growth promotion were tested *in vitro*: nitrogen fixation, phosphate solubilization, siderophore production, and indole-3-acetic acid (IAA) production. Presumptive nitrogen fixation was qualitatively evaluated by growing the PGPR in the nitrogen-free semisolid medium (JNFb) as described by Olivares et al. (1996). Phosphate solubilizing capacity was qualitatively evaluated by the plate assay using National Botanical Research Institute's phosphate growth medium (NBRIP) which contained calcium phosphate as the inorganic source of phosphate (Nautiyal, 1999). Siderophore production was qualitatively evaluated by Chrome Azurol S medium (Alexander and Zuberer, 1991). IAA production was assayed by the quantitative analysis using ferric chloride-perchloric acid reagent ($FeCl_3$-$HClO_4$) (Gordon and Weber, 1951). Each of these tests was repeated three times.

The induction of systemic resistance to black rot was tested *in planta*. Kaboko Hybrid Organic Chinese Cabbage Seeds (Park Seed, USA) were planted in germination trays containing 25 cm^2 holes. One ml of PGPR spore suspension (10^7 CFU/ml) was applied to each seed prior to covering with potting medium. Seeds were put into the germination incubator (28 ℃) for two days, and then placed in the greenhouse. Fourteen days after seeding, cabbage seedlings were transplanted into 4.5-inch round pots. Each pot was drenched with 50ml of a PGPR spore suspension (10^6 CFU/ml) at transplanting time. Freshly prepared suspensions of *X. campestris* pv. *campestris* were sprayed onto the leaves two weeks after soil drench. Pathogen-inoculated plants were placed into the dew chamber (100% humidity) under darkness for two days at 24 ℃, and then moved to a greenhouse at 32/25 ℃ day/night. Plants were watered daily.

Fourteen days after pathogen challenge, total lesion number (TLN) was recorded for each plant. Plants were harvested at the same time and the following plant parameters were measured: plant diameter, shoot fresh weight (SFW), shoot dry weight (SDW) (oven dry at 90 ℃), root fresh weight (RFW), and root dry weight (RDW) (oven dry at 90 ℃). The experiment was arranged as a randomized complete block design (RCBD), with a single cabbage per pot for eight replications per treatment. The experiment was repeated once.

2.4. Advanced test of selected individual stains and mixtures of selected stains in the greenhouse and field

Four individual PGPR strains (AP136, AP209, AP282, and AP305) selected in the preliminary screening were tested again. These four strains that showed the induced systemic resistance were mixed together as mixture-1 (AP136, AP209, AP282, and AP305), and three strains (AP7, AP18, and AP218) that showed growth promotion were added to mix-1 for mixture-2 (AP7, AP18, AP136, AP209, AP218, AP282, and AP305). A total of seven treatments were used: four

treatments consisting of single PGPR strains (AP136, AP209, AP282, and AP305), two treatments consisting of strain mixtures (mixture-1 and mixture-2), and one control (a nonbacterized but pathogen-challenged disease). The methods were the same as previously described. The mixture of PGPR strains were prepared by combining equal proportions of each strain prior to application to the seed. The experiment was conducted twice.

The same treatments were tested once at E.V. Smith Research Center, Shorter, AL, USA (32.45 N, 85.88 W). The soil type is sandy loam. The total amount of rainfall during this experiment was 116 mm. The maximum average temperature ranged from 17.17 ℃ to 24.44 ℃, while the minimum varied form 2.11 ℃ to 10.17 ℃. Seedlings were transferred into the field after growing for six weeks in the greenhouse, and each plant was drenched with 100 ml of PGPR spore suspension (10^6 CFU/ml) at the transplanting time. Two weeks after inoculation with the PGPR, the whole plant was sprayed with the pathogen (10^8 CFU/ml). The inoculations of PGPR and pathogen were done in the late afternoon to prevent rapid drying. This experiment was laid out in a randomized complete block design, and each treatment had six plots. Each plot contained 10 plants (2 rows of 5 plants each), and the distance between rows was 60 cm and within rows was 45cm.

The biological control effect was quantified by recording disease incidence at three and ten weeks after transplanting, and assessing the external, head surface, and internal black rot symptoms 71 days after transplanting. For scoring the external black rot index (EBR index), leaves not forming part of the head were examined for black rot symptoms and assessed as follows: EBR index =(0a + 1b + 2c + 3d + 4e)/T, where: 0, 1, 2, 3 and 4 indicate respectively none, >0–10%, 11–20%, 21–30% and >30% of the surface of a leaf showing black rot symptoms; a–e correspond to the number of leaves in the infection category; T is the total

number of external leaves. For recording the head index (HBR index), the surface of the whole head was checked as follows: HBR index = (0a + 1b + 2c + 3d + 4e)/T, where a=no symptom on the head surface; b=the symptoms only on the surface; c= symptoms on the surface and inside of leaf; d= symptoms on the surface and inside of leaf, and form V-shape lesion; e= symptoms on the surface and inside of leaf, form V-shape lesion and veil discoloration. For assessing the internal black rot index (IBR index), cabbage heads were cut perpendicularly into quarters and the internal symptoms were assessed as follows: 0 =No discoloration, no symptoms on the heart leaves (healthy plants); 1 = vein discoloration extends <1/2 of the stem, no symptoms on the heart leaves; 2 = vein discoloration extends >1/2 of the stem, no symptoms on the heart leaves; 3 = vein discoloration of stem and 1–3 of the heart leaves and 4 = vein discoloration of stem and on more than 3 heart leaves.

To determine the yield, the whole plant was recorded as the whole yield. After peeling off the leaves that did not form the head, the weight was recorded as the head yield. The marketable yield was the head after removing any outer leaves with symptoms.

2.5. Statistical analysis

All data were analyzed by analysis of variance (ANOVA), and the treatment means were separated by using Fisher's protected least significant difference (LSD) test at P=0.05 using SAS 9.4 software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Preliminary screening

Ten PGPR strains (AP7, AP18, AP136, AP188, AP209, AP213, AP218, AP219, AP295, and AP305) showed presumptive nitrogen fixation capacity (Table 1). Eight PGPR strains (AP136, AP188, AP209, AP213, AP218, AP219, AP295, and AP305) positively produced siderophores.

None of the PGPR strains produced a halo on NPRIP medium. All 12 of the tested PGPR strains produced IAA at levels of 2.11-14.95 μg/ml.

For induced systemic resistance *in planta*, total lesion number was significantly reduced by ten strains (AP136, AP188, AP209, AP213, AP217, AP218, AP219, AP282, AP295, and AP305) compared to control (Table 2). Treatments AP7, AP18, AP136, AP218, and AP295 increased fresh shoot weight, dry shoot weight, fresh root weight, and plant diameter. Treatments AP282 and AP305 increased the fresh root weight, and treatments AP213 and AP305 increased the plant diameter compared with the control.

3.2. Advanced test by selected individual stains and mixtures of selected stains in the greenhouse and field

Treatments (AP136, AP209, AP 282, mixture-1 and mixture-2) significant reduced the total lesion number, increased the shoot fresh weight and root fresh weight in the greenhouse test (Table 3). The plant diameter was increased by treatments (AP136, AP209, AP305 and mixture-1). Only mixtures-2 significantly increased the shoot dry weight and root dry weight.

Three weeks after transplanting to the field, all the PGPR treatments significant delayed the pathogen infection (Table 4). At harvest time, treatments of AP136, AP209, AP305 and mixture-1 significantly reduced disease incidence compared to control. Although the biocontrol effect of all the treatments was not found to be significant in cabbage leaves (EBR index), treatments AP136 reduced the severity of black rot on cabbage leaves (EBR index). All the treatments significantly reduced the black rot symptoms on cabbage head (Head index) compared with the control. At harvest, no internal black rot symptoms were observed inside of the cabbage head (IBR Index). For the yield, no statistically significant effects on whole yield and head yield were observed. However, all the PGPR treatments significantly increased the marketable yield.

4. Discussion

The results presented here confirmed that individual PGPR strains and strain mixtures induced systemic resistance to black rot in the greenhouse and field, and the marketable yield was increased by individual and mixtures of PGPR strains.

Reduction of total lesion numbers and increase of shoot fresh weight and root fresh weight by PGPR-mediated ISR in the greenhouse, and reduction of disease incidence three weeks after transplanting, head black rot index at harvest and increase the marketable yield were highly correlated, suggesting that plant growth promotion may be partially due to inhibition of infection by the pathogen or reduction of its development in the plant (Table 3 and 4). Previously, ISR elicited by PGPR was reported to result in defense against pathogen spread within the plant (Ahemad and Kibret, 2014; Liu et al., 1995). Some *Bacillus* spp. PGPR that elicited ISR also promoted plant growth (Kloepper et al., 2004; Wei et al., 1996; Zhang et al., 2004). However, these previous studies did not test whether plant growth was caused by the direct mechanisms of plant growth promotion. In the current study, traits related to plant growth promotion were tested, including biological nitrogen fixation, solubilization of mineral phosphate, secretion of plant hormones, and siderophore production. Eight of 12 tested strains exhibited three traits related with plant growth promotion. Ten strains showed presumptive nitrogen fixation. Future work is needed to confirm nitrogenase activity by gas chromatography using the C_2H_2 reduction technique (Hardy et al., 1973). In the advanced test, the selected individual PGPR strains (AP136, AP209 and AP305) and strain AP218 included in mixture-2 showed positive activities for putative nitrogen fixation, IAA production, and siderophore production that have been directly related to plant growth promotion (Table 1). In addition, strains AP7 and AP18, which are included in mixture-2, were positive for putative nitrogen fixation and IAA production. In

previous reports, a single PGPR strain causing biological control and growth promotion through multiple mechanisms was also demonstrated (Ryu et al., 2004; Ryu et al., 2003; Wahyudi and Astuti, 2011).

Some previous studies on biological control of black rot focused only on biocontrol effects without evaluating growth parameters in the greenhouse or yield in the field (Mishra and Arora, 2012b; Wulff et al., 2002). However, ideal biocontrol agents should have positive effects on the yield. In one study that recorded yield at harvest, treatments did not significantly increase yield, and some of the tested *Bacillus* strains even caused a significant reduction in cabbage yield (Massomo et al., 2004). In the current study, all the tested PGPR treatments significantly increased marketable yield in the field (Table 4), and, except for strain AP305, promoted shoot and root fresh weights in the greenhouse (Table 3). These results are in agreement with previous reports of increases in several plant growth parameters when induced systemic resistance was demonstrated (Wei et al., 1991). Therefore, these tested PGPR treatment may not only cause the higher consumer prices by increasing the quality through biocontrol effect, but also increase the total income due to the growth promotion.

One criterion for ISR by PGPR is the physical separation of tested bacteria from the target pathogens. In this research, the PGPR were introduced by seed treatment and root drench, and the pathogen was inoculated by the foliar spray. Hence, the PGPR and pathogen were separated spatially and therefore, the reduction of the disease was due to ISR.

Several studies have demonstrated that mixtures of PGPR strains can be more effective due to synergistic modes of action (Jetiyanon and Kloepper, 2002; Raupach and Kloepper, 1998). In this study, we formed two mixtures based on results of the preliminary screening, including mixture-1 that contained 4 PGPR strains (AP136, AP209, AP282, and AP305) that elicited

induced systemic resistance and mixture-2 that contained mixture-1 and 3 other PGPR strains (AP7, AP18, and AP218) that exhibited growth promotion. Mixture-1 reduced disease incidence three weeks after transplanting at a greater level than did some individual PGPR treatments in the field (Table 4). Mixture-2 elicited the largest growth promotion compared with other treatments, significantly increasing root and shoot dry weights in the greenhouse (Table 3). Although mixtures did not exhibit the greatest growth promotion in the field, they still increased the marketable yield. Mixtures of PGPR can have other benefits compared to individual PGPR strains. For example, mixtures have demonstrated increased repeatability of efficacy over multiple field trials (Jetiyanon et al., 2003; Raupach and Kloepper, 1998). In addition, mixtures of several strains may result in a more stable rhizosphere community and provide several mechanisms under field conditions (Domenech et al., 2006). In the future, the PGPR treatments used in the current study should be evaluated in multiple field conditions, to test their consistency.

Strains AP7 (previously SE52) and AP18 (previously INR7) did not reduce black rot of cabbage in this study (Table 2). However, strain INR7 (AP18) showed the capacity to elicit systemic protection on cucumber against *Erwinia tracheiphila* (Zehnder et al., 2001), on cucumber against to *P. syringae* pv. l*achrymans* (Wei et al., 1996), on tomato against to *Ralstonia solanacearum*, on long cayenne pepper against to *Colletotrichum gloeosporioides* (Jetiyanon and Kloepper, 2002), on cucumber against to *C. gloeosporioides* (Raupach and Kloepper, 1998), and strains INR7 (AP18) and SE52 (AP7) elicit systemic protection on loblolly pine against to *Cronartium quercuum* (Enebak et al., 1998). It appears that the broad-spectrum protection resulting from PGPR-ISR can be strain specific. However, the growth promotion activity of INR7 and SE52 in our tests agree with previous reports of enhanced germination rate of loblolly

and slash pine by both PGPR strains (Enebak et al., 1998), increased weight of slash pine shoots by INR7 (Enebak et al., 1998), and increases in cucumber main runner length and number of leaves by INR7 (Wei et al., 1996).

In summary, the results reported here showed that PGPR-mediated ISR not only protected cabbage against black rot but also increased growth parameters in the greenhouse and field conditions. In addition to using a single PGPR strain, it may be possible to apply mixtures of PGPR strains. In future, several additional issues should be addressed, including the length of protection and the consistency of mixtures of PGPR. PGPR-mediated ISR should be further evaluated in an integrated pest management approach for controlling black rot of cabbages.

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Table 1. Presumptive nitrogen fixation, phosphate solubilization, siderophore production, and indoleacetic acid.

Strain[a]	ID	Presumptive nitrogen fixation	Phophate solubiliazation	Siderophore production	IAA production (μg/ml)
AP7	*Bacillus safensis*	+	−	−	2.28
AP18	*Bacillus altitudinis*	+	−	−	2.66
AP136	*Bacillus amyloliquefaciens*	+	−	+	6.51
AP188	*Bacillus amyloliquefaciens*	+	−	+	6.65
AP209	*Bacillus mojavensis*	+	−	+	4.93
AP213	*Bacillus amyloliquefaciens*	+	−	+	8.19
AP217	*Bacillus solisalsi*	−	−	−	3.24
AP218	*Bacillus methylotrophicus*	+	−	+	8.65
AP219	*Bacillus methylotrophicus*	+	−	+	10.16
AP282	*Lysinibacillus boronitolerans*	−	−	−	2.11
AP295	*Bacillus amyloliquefaciens*	+	−	+	6.51
AP305	*Bacillus amyloliquefaciens subsp. plantarum*	+	−	+	14.95
Control		−	−	−	1.42

[a]+ = Positive for presumptive nitrogen fixation, phophate solubiliazation, siderophore production, − = Negative for presumptive nitrogen fixation, phophate solubiliazation, siderophore production

Table 2. Results of preliminary screen of PGPR for induction of systemic resistance (ISR) against black rot.

Treatments[ab]	Total lesion number	Shoot fresh weight(g)	Shoot dry weight(g)	Root fresh weight(g)	Root dry weight(g)	Plant diameter (cm)
Control	9.44 a	19.93 efg	1.00 ef	0.32 efd	0.02 b	28.63 ef
AP7	8.93 ab	36.98 ab	1.73 abc	0.75 ab	0.04 b	34.57 abcd
AP18	8.13 abc	45.68 a	2.17 a	0.90 a	0.04 b	35.56 abc
AP136	5.31 d	32.26 bcd	1.62 bcd	0.59 bc	0.02 b	33.44 bcd
AP188	5.88 cd	13.26 g	0.78 f	0.28 f	0.01 b	23.88 g
AP209	6.25 cd	16.61 fg	0.92 ef	0.31 ef	0.02 b	24.84 fg
AP213	6.29 cd	27.62 bcde	1.37 bcde	0.54 bcd	0.01 b	33.21 bcd
AP217	6.56 bcd	22.71 defg	1.18 def	0.46 cdef	0.03 b	28.81 ef
AP218	6.81 bcd	36.33 ab	1.83 ab	0.65 bc	0.03 b	38.00 a
AP219	6.00 cd	28.92 bcde	1.43 bcde	0.53 bcde	0.02 b	32.15 cde
AP282	5.94 cd	28.43 bcde	1.37 bcde	0.60 bc	0.15 a	30.63 de
AP295	6.50 bcd	34.41 bc	1.65 bcd	0.63 bc	0.02 b	36.63 ab
AP305	5.69 cd	25.14 cdef	1.24 cdef	0.61 bc	0.02 b	34.15 abcd
LSD $_{0.05}$	2.5	11.01	0.51	0.22	0.10	4.36

[a]The experiment was arranged by randomized completely block design (RCBD), using 4.5-inch round pots with single cabbage per pot for eight replications per treatment. The experiment was repeated once. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]PGPR strains applied as seed treatment and soil drench. Strain AP7= *B. safensis*, AP18= B. *altitudinis*, AP209= *B. mojavensis*, AP214= *B. amyloliquefaciens*, AP217= *B. solisalsi*, AP282= *Lysinibacillus boronitolerans*, AP305= *B. amyloliquefaciens* subsp. *plantarum*, AP136, AP188, AP218, AP219, and AP295=*B. methylotrophicus*. A nonbacterized but pathogen-challenged treatment was used as the control

Table 3 Results of advanced test by selected individual strains and mixtures of selected strains for induction of systemic resistance (ISR) against black rot in the greenhouse.

Treatments[ab]	Total lesion number	Shoot fresh weight(g)	Shoot dry weight(g)	Root fresh weight(g)	Root dry weight(g)	Plant Diameter (cm)
Control	20.35 a	29.96 b	2.59 b	5.94 d	0.58 bc	31.20 c
AP136	15.82 b	37.29 a	2.80 ab	7.00 c	0.50 bcd	33.44 ab
AP209	17.19 b	35.59 a	2.65 b	7.32 bc	0.36 d	33.87 ab
AP282	16.03 b	35.66 a	2.87 ab	8.18 b	0.52 bc	32.29 bc
AP305	17.96 ab	34.72 ab	2.86 ab	6.64 cd	0.47 cd	34.58 a
Mixture-1	17.58 b	37.28 a	2.89 ab	8.20 b	0.63 b	33.98 ab
Mixture-2	16.58 b	39.04 a	3.03 a	10.04 a	0.85 a	33.22 abc
LSD0.05	2.44	4.92	0.35	1.04	0.15	2.07

[a]The experiment was arranged by randomized completely block design (RCBD), using 4.5-inch round pots with single cabbage per pot for eight replications per treatment. The experiment was repeated twice. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]PGPR strains applied as seed treatment and soil drench. Mixture-1: AP136, AP209, AP282 and AP305; Mixture-2: AP136, AP209, AP282, AP305, AP213, AP7, AP18 and AP218.

Table 4. Effects of individual plant growth-promoting rhizobacteria (PGPR) strains and strain mixtures on incidence and severity of black rot disease and yield in the field.

Treatments[ab]	Disease incidence		Disease severity			Yield[c]		
	3 weeks after transplanting	10 weeks after transplanting	External black rot index	Head black rot index	Internal black rot index	Whole yield	Head Yield	Market Yield
Disease	45.0 a	78.3 a	1.88 a	2.65 a	0	38.13 bc	29.64 ab	21.11 d
AP136	6.7 d	43.3 b	1.84 a	1.03 c	0	38.29 bc	29.59 ab	25.30 ab
AP209	25.0 bc	41.7 b	1.94 a	1.18 bc	0	38.47 b	28.75 b	22.51 c
AP282	23.3 bc	61.7 ab	1.96 a	1.45 bc	0	42.05 a	30.27 a	24.56 b
AP305	20.0 bc	51.7 b	2.16 a	1.23 bc	0	39.48 b	29.91 ab	25.86 a
Mixture-1	18.3 c	56.7 b	1.96 a	1.18 bc	0	38.92 b	27.40 c	23.29 c
Mixture-2	30.0 b	58.3 ab	2.09 a	1.22 bc	0	36.32 c	26.49 c	22.52 c
LSD0.05	10.4	21.5	0.32	0.38	0	1.98	1.34	1.21

[a] The experiment was arranged as a randomized complete block design, and each plot contained 10 plants (2 rows of 5 plants each). The experiment was conducted once. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.

[b]PGPR strains applied as seed treatment and soil drench. Mixture-1: AP136, AP209, AP282 and AP305; Mixture2: AP136, AP209, AP282, AP305, AP213, AP7, AP18 and AP218.

[c]For the plant growth, the whole plot was recorded as the whole sampled plants. After peel off the leaves that did not form the head part, the yield was recorded as the head yield. The marketable yield was the head without any symptoms.

Figure 1. Key traits of plant growth promotion *in vitro*. (A) Growth pellicle formation by presumable N-fixing isolates (arrows) in semi-solid N-free culture medium, AP7 (*B. safensis*); (B) Orange halo around the colony of AP 219 (*B. amyloliquefaciens*) indicating the ability of this isolate to excrete siderophores that removed Fe from Fe-CAS agar medium.