INDUCTION OF GROWTH PROMOTION AND STRESS TOLERANCE IN ARABIDOPSIS AND TOMATO BY PLANT GROWTH-PROMOTING RHIZOBACTERIA

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INDUCTION OF GROWTH PROMOTION AND STRESS TOLERANCE IN ARABIDOPSIS AND TOMATO BY PLANT GROWTH-PROMOTING RHIZOBACTERIA

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DISSERTATION ABSTRACT

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Plant growth-promoting rhizobacteria (PGPR) were evaluated for induction of growth promotion in Arabidopsis and enhancement of stress tolerance in tomato under greenhouse and field conditions. *Bacillus* strain GB03 not only resulted in larger plant size, but significantly enhanced Arabidopsis growth rate and shortened the time to attain each growth stage from the three-leaf stage to inflorescence emergence. Elicitation of Arabidopsis growth promotion by PGPR is most effective during early stages of development. However, induced systemic resistance by PGPR may vary according to plant species and PGPR strain.

The interaction between PGPR strain and Arabidopsis root morphology was conducted in a Petri dish system. *Bacillus* PGPR strains reduced Arabidopsis total root length but enhanced root hair length in a dose-dependent manner. The mechanism of

bacilli elicitation of growth promotion may involve the enhancement of root hair development and therefore increase nutrient and water uptake.

Tomato was used to study the effect of PGPR on plant growth under different environmental stresses. The effect of PGPR on tomato growth under salt stress was conducted under greenhouse conditions. Some *Bacillus* PGPR strains ameliorated tomato emergence, shoot growth and chlorophyll content under lower levels of salt stress. However, PGPR have little or no effect on tomato root growth under salt stress. Moreover, induction of tomato salt stress tolerance by PGPR is strain-specific. Application of PGPR to enhance stress tolerance in plants is a feasible strategy for improving crop production in saline environments.

The effect of PGPR on tomato growth, yield, and fruit quality was conducted under field stresses in northeast Alabama. Commercially available PGPR products can lessen the stress of transplant shock and nitrogen stress resulting from organic fertilizer. PGPR treatments consistently resulted in significantly higher plant growth indices compared to nonbacterized control. With organic fertilizer, tomato fruits had significantly higher sugar and vitamin C contents compared to tomato fruits grown with inorganic fertilizer. Although marketable yield was less with organic fertilizer, fruit quality was higher. Moreover, some PGPR products in combination with organic fertilizer contributed to the improvement of tomato flavor quality and nutrient quality.

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I. INTRODUCTION

1. Plant growth-promoting rhizobacteria

The rhizosphere – the zone surrounding the root system of all plants where exudates stimulate microbial growth – has a major influence on the health and productivity of crops. Among rhizosphere microorganisms, some can reduce plant growth by acting as pathogens, while others such as plant growth-promoting rhizobacteria (PGPR) colonize roots and can promote plant growth (Kloepper, 1994). PGPR can promote plant growth directly through nitrogen fixation (Dobbelaere et al., 2003; Glick et al., 1999), facilitation of nutrient uptake (Biswas et al., 2000; Dobbelaere et al., 2003), solubilization of phosphorus (Richardson, 2001; Rodriguez and Fraga, 1999), phytohormone production (Persello-Cartieaux et al., 2003), or by lowering soil levels of ethylene (Glick et al., 1998; Stearns and Glick, 2003). In addition, PGPR may indirectly promote plant growth by decreasing or preventing the effects of nonpathogenic, deleterious microorganisms through production of antimicrobial compounds (Handelsman and Stabb, 1996; Raaijmakers et al., 2002), production of siderophores which helps them compete with other microbes (including pathogens) for iron (Crowley et al., 1991; Wang et al., 1993), competition for colonization sites on the root, competition for nutrients (Buysens et al., 1996; Lugtenberg et al., 2001), or induced systemic resistance (Bakker et al., 2003; van Loon et al., 1998).

In addition to promoting plant growth, PGPR can also act as antagonists to suppress the activity of soilborne pathogens. The modes of action of disease suppression by PGPR include synthesis of antibiotics or other antimicrobial compounds such as hydrogen cyanide (Hass et al., 2002); competition for substrates or infection sites (Lugtenberg et al., 2001); secretion of cell wall-degrading enzymes such as chitinase and glucanase (Whipps, 2001); and induced systemic resistance (Bakker et al., 2003; van Loon et al., 1998). The growth-promoting and disease-suppressive effects of PGPR often cannot be separated. The mechanisms accounting for growth promotion or biological control vary among PGPR species and even among strains of a given species of PGPR (van Loon and Bakker, 2003). In addition, more than one mechanism of a PGPR strain may be utilized depending on the plant species colonized (Handelsman and Stabb, 1996; Whipps, 2001).

The principle rhizobacterial genera known to act as PGPR include *Azospirillum* (Bashan, 1999; Okon, 1994), *Bacillus* (Emmert and Handelsman, 1999), *Burkholderia* (Coventry and Dubery, 2001), *Enterobacter* (Nie et al., 2002), *Paenibacillus* (Timmusk et al., 1999), *Pseudomonas* (Walsh et al., 2001), *Serratia* (Press et al., 2001; Press et al., 1997), and *Streptomyces* (Emmert and Handelsman, 1999). These rhizobacteria have been studied for their potential as biocontrol agents to control plant diseases and as biofertilizers to improve plant growth (Lucy et al., 2004).

The largest number of reports of PGPR involves *Pseudomonas* spp. The pseudomonads rapidly colonize roots and produce several different antifungal metabolites and, therefore, have been widely applied as biocontrol agents (Walsh et al., 2001). While there are fewer reports using bacilli as PGPR, this group has the practical

advantage of producing heat- and desiccation-tolerant endospores. Endospores allow formulation of the bacilli into commercial products for biofertilizers or biocontrol agents (Emmert and Handelsman, 1999; Handelsman and Stabb, 1996). Despite early attempts to produce formulations of pseudomonads, almost all PGPR products currently produced in the U.S. contain bacilli with a long shelf life. A growing number of PGPR are being commercialized as biocontrol agents or biofertilizers in the U.S. (Glick et al., 1999; Kloepper et al., 2004b; McSpadden Gardener and Fravel, 2002). One of the major challenges for large-scale application of PGPR products is to maintain high populations of living microorganisms for longer shelf-life. Several commercially available biocontrol products, such as Kodiak®, Serenade®, and Yield Shield®, include *Bacillus* spp. which form desiccation-resistant and heat-resistant endospores that facilitate the formulation processes.

The ultimate goal of Integrated Pest Management (IPM) is to use multiple cost-effective strategies for disease management and minimize environmental damage to achieve sustainable agricultural production (Cook, 2000). PGPR are good components to use in IPM because their application in agriculture can increase crop yield. In addition, the input of pesticides for disease control, which often accumulate in the ecosystem and are hazardous to animals and humans, can be reduced by inoculating PGPR as biocontrol agents.

2. Induced resistance

Plant resistance to disease is regulated at the cultivar level by vertical resistance genes. According to the gene-for-gene concept, resistance occurs when a vertical

resistance gene in the host encounters a corresponding avirulence gene in the pathogen, leading to recognition of the pathogen by the host. Genetic resistance to disease may also occur in plants as a result of horizontal resistance that is mediated by multiple host resistance genes. Plants that contain vertical or horizontal resistance genes are considered to be genetically resistant (Agrios, 1997).

Susceptible plants – those lacking genetic resistance to a pathogen – still develop defense reactions in response to infection by a pathogen. However, activation of such host defense typically occurs too late in the infection process to stop disease development. If host defense could be triggered prior to pathogen infection, then disease incidence or severity could be reduced. This phenomenon was first recognized in 1901 by Ray and Beauverie, when they used hypovirulent strains of *Botrytis cinerea* to protect plants from subsequent infection by highly virulent strains (Kessmann et al., 1994). The general phenomenon of such protection is termed induced resistance. Induced resistance can be local when protection results only at the inoculated site or systemic when the protection is seen throughout the plant. Induced systemic resistance has been referred to in the literature as systemic acquired resistance (SAR) (Sticher et al., 1997) and induced systemic resistance (ISR) (van Loon et al., 1998).

Both SAR and ISR are expressed against a broad spectrum of pathogens and have been demonstrated in a wide range of plant species, including monocots and dicots (Sticher et al., 1997; van Loon et al., 1998). SAR and ISR do not prevent the disease from occurring but reduce the severity of disease. Thus, induced resistance can enhance general plant resistance.

3. Systemic acquired resistance (SAR)

Systemic acquired resistance (SAR) can be induced by virulent pathogens, avirulent forms of pathogens, incompatible races of pathogens, or certain chemicals (Metraux, 2001), all of which lead to the hypersensitive response (HR) (Gilchrist, 1998; Grant and Mansfield, 1999; Lamb and Dixon, 1997; Morel and Dangl, 1997). In the HR, localized cell death occurs in plant cells surrounding the pathogen. As a result, infection is blocked. The mechanisms involved in SAR include modification of cell wall structure, like lignificaiton and other structural barriers (Hammerschmidt, 1999a), synthesis of phytoalexins (Hammerschmidt, 1999b), and production of pathogenesis-related proteins (PRs) that have shown some antimicrobial activities *in vitro* (Hunt and Ryals, 1996; van Loon, 1997; van Loon and van Strien, 1999).

Salicylic acid (SA) acts as a systemic signal molecule during the SAR signaling pathway and usually enhances expression of PRs in the induced plant. The essential role of SA in SAR was shown in transgenic plants that carry a bacterial salicylate hydroxylase (*nahG*) gene (Delaney et al., 1994; Gaffney et al., 1993). In both *NahG* transgenic tobacco and Arabidopsis, SA did not accumulate after pathogen infection, and SAR was not elicited (Bi et al., 1995; Friedrich et al., 1995; Gaffney et al., 1993; Lawton et al., 1995).

4. Induced systemic resistance (ISR)

Kloepper et al. (1992) described the phenomenon of induced systemic resistance (ISR) as being elicited by specific rhizobacteria, such as PGPR. ISR by these rhizobacteria was first reported in carnation against *Fusarium oxysporum* (van Peer et al.,

1991) and in cucumber against *Colletotrichum orbiculare* (Wei et al., 1991). In these studies, there was no physical contact between PGPR strains, which colonized roots, and the pathogens, which were inoculated on above-ground plant parts. Thus, disease suppression resulted from a host-mediated phenomenon induced by PGPR rather than from antagonism. ISR has now been demonstrated in *Arabidopsis thaliana*, bean (*Phaseolus vulgaris*), carnation, cucumber (*Cucumis sativus*), radish, tobacco (*Nicotiana tabacum*), and tomato against fungi, bacteria, viruses, and insects (van Loon and Bakker, 2003; van Loon et al., 1998).

In contrast to SAR, ISR elicited by PGPR is not associated with the hypersensitive reaction (HR), and SA may or may not be involved (Park and Kloepper, 2000; Press et al., 1997). Other signal components are more commonly involved in ISR signaling, such as jasmonic acid (JA) and ethylene. Also, no PR proteins are induced during ISR in Arabidopsis (Pieterse et al., 1996).

Although ISR enhances the general resistance in the plant, different PGPR strains induce resistance differentially in different plant species (van Loon, 1997; van Loon and Glick, 2004). For example, three *Pseudomonas* spp. PGPR strains – WCS358 , WCS374, and WCS417 – can elicit ISR against *F. oxysporum* in crucifers. However, WCS358 induced ISR in Arabidopsis but not in radish; WCS374 induced ISR in radish but not in Arabidopsis; and WCS417 induced ISR in both Arabidopsis and radish (van Loon and Bakker, 2003). On the other hand, even in the same host, the same PGPR strain induces resistance differentially against the same pathogen but different pathovars. Ryu et al. (2003b) found that *Paenibacillus polymyxa* strain E681 only induced resistance against *Pseudomonas syringae* pv. maculicola but not against *P. syringae* pv. tomato. This

difference was attributed to different signaling pathways of induced resistance by these PGPR strains. The signaling pathway of *Bacillus pumilus* SE34 that induced resistance against *P. syringae* pv. tomato was dependent on SA, while the pathway against *P. syringae* pv. maculicola was SA-independent (Ryu et al., 2003b). Two PGPR strains, belonging to diverse bacterial genera, *P. fluorescens* 89B61 and *B. pumilus* T4, have shown protection against these pathogens in jasmonic acid deficient and ethylene insensitive Arabidopsis. Thus, resistance was possibly induced via a new pathway that has not been reported previously. These results suggest that induced resistance by PGPR in the same plant species is PGPR strain specific and pathogen specific, and may use a different mechanism against different pathogens.

The precise role of bacterial determinants in elicitation of ISR is unclear. Several bacterial determinants or factors, such as lipopolysaccharides (LPS) (Leeman et al., 1995; van Peer and Schippers, 1992) and siderophores (Leeman et al., 1996; Maurhofer et al., 1994; van Loon et al., 1998), have been shown to be important for elicitation of ISR. Some PGPR strains can produce SA in iron-limiting soils, and bacterial production of SA may be a main determinant for ISR elicitation by these strains (de Meyer et al., 1999; de Meyer and Höfte, 1997).

5. *Arabidopsis thaliana* as a model system for studying plant-microbe interactions

Arabidopsis thaliana belongs to the mustard family (Brassicaceae) and has a broad natural habitat throughout Europe, Asia, and North America (Meinke et al., 1998). A large number of ecotypes have been collected from natural populations and are accessible for experimental studies. The Columbia and Landsberg ecotypes are

commonly used for genetic and molecular research. The advantages of using Arabidopsis for basic plant molecular biology study include; a rapid life cycle of about 6 weeks from germination to mature seeds; small size, allowing growth in Petri dishes or pots in limited space; self-fertility; a small genome size that is completely sequenced; production of several hundred siliques – the long two-valved seed vessel or pod – with more than 5,000 total seeds; and availability of numerous ecotypes and mutant lines (TAIR: www.arabidopsis.org).

The signaling pathways for both SAR and ISR have been reported mostly in Arabidopsis as a model system (Glazebrook, 2001; Pieterse et al., 1998; Thomma et al., 2001). The numerous metabolic mutants available in Arabidopsis make it a powerful tool for studying signal transduction and their interaction or cross-talk.

6. Regulation of plant ethylene levels by PGPR

Ethylene is a simple gaseous hydrocarbon that regulates many physiological processes, including seed germination, root and shoot growth, flower development, senescence of plant organs, and ripening of fruit (Bleecher and Kende, 2000). Ethylene is also involved in the modulation of plant responses to a wide variety of biotic and abiotic stresses. When plants are subject to different environmental or biological stresses, they typically increase biosynthesis of ethylene. For example, mechanical wounding, drought, flooding, heat, chilling, salinity, and oxidative stresses can induce ethylene production in plants (Morgan and Drew, 1997).

In higher plants, ethylene is produced from its precursor

1-aminocyclopropane-1-carboxylic acid (ACC) by ACC oxidase (John, 1997; Yang and Hoffman, 1984). In soil, some PGPR, such as *Enterobacter cloacae* UW4 and CAL2 (Grichko and Glick, 2001a) and *Pseudomonas putida* GR12-2 (Mayak et al., 1999), can synthesize the enzyme ACC deaminase, which degrades ACC, as their nitrogen source. Glick et al. (1998) proposed a model for lowering plant ethylene levels by ACC deaminase-containing PGPR. Ethylene is required to break seed dormancy and stimulate germination, but a sustained high level of ethylene in the plant could inhibit root elongation after germination. When ACC deaminase-containing PGPR are bound to seeds or roots of seedlings, they could reduce the plant ethylene level and its inhibition of root elongation. By promoting the longer roots, these bacteria may improve seedling survival, especially during the first few days after seeding. The capacity of promoting root elongation was greatly diminished when ACC deaminase mutants of *E. cloacae* UW4 and *P. putida* GR12-2 were applied compared to wild-type strains (Glick et al., 1994; Li et al., 2000). In addition, ACC deaminase-containing PGPR reduce the deleterious effects of stress ethylene when treated plants are subjected to environmental stresses such as flooding (Grichko and Glick, 2001b), heavy metals (Belimov et al., 2005; Burd et al., 1998), fungal pathogens (Glick et al., 1999), drought, and high salt (Mayak et al., 2004a; b).

Ethylene-induced senescence results in large losses of fruits and vegetables annually. In order to prevent economic losses in agriculture, it is important to lower ethylene levels in the plant or its fruit (Arshad and Frankenberger, 2002). Several chemical inhibitors of ethylene have been widely used to lower ethylene levels (Biles et al., 1990; Robison et al., 2001), but these chemicals are potentially hazardous to the

environment and animals. Several transgenic plants with altered endogenous levels of ethylene have been created in Arabidopsis, broccoli, canola, cantaloupe, carnation, melon, petunia, tobacco, tomato, and potato (Stearns and Glick, 2003). Transgenic tomato plants with bacterial ACC deaminase have shown protection against several phytopathogens (Lund et al., 1998; Robison et al., 2001). When an ACC deaminase gene was introduced into an antibiotic-producing biocontrol agent, *P. fluorescens* strain CHA0 (Schnider et al., 1995), the transformed bacterium increased root length of canola plants and improved its capacity to protect cucumber against *Pythium* damping-off and potato tubers against *Erwinia* soft rot (Wang et al., 2000). In conclusion, lowering plant ethylene levels by ACC deaminase-containing PGPR can promote plant growth, reduce the deleterious effects of both biotic and abiotic stresses, and act synergistically with other mechanisms of biocontrol to suppress plant diseases (van Loon and Glick, 2004).

7. Enhanced tolerance to environmental stresses by PGPR

Environmental stresses are limiting factors for agricultural productivity worldwide. These stresses not only decrease the yield of crops but also represent barriers to the introduction of crop plants into areas that are not suitable for crop cultivation. Abiotic stress factors include high and low temperatures, salinity, drought, flooding, ultraviolet light, heavy metals, and oxidative stresses (Nilsen and Orcutt, 1996).

Heat stress is one of the more important constraints of crop production, and its frequency is increasing due to global warming (Iba, 2002; Rosenzweig and Parry, 1994). At high temperature, photosynthesis is inhibited, carbohydrate reserves decline, and crop yields are reduced. When plants are exposed to excess heat, a characteristic set of cellular

and metabolic responses is triggered. The heat stress or heat-shock response is characterized by a transient expression of heat-shock proteins (HSPs) (Iba, 2002). HSPs have been identified in microorganisms, animals (including humans), as well as plants. The functions of all the different HSPs are not clearly known, but some of them act as molecular chaperones that bind to partially folded or denatured proteins and thereby prevent irreversible aggregation. Some HSPs assist polypeptide transportation into subcellular compartments, such as chloroplasts and mitochondria. These interactions between polypeptides and HSPs may be especially critical at high temperatures because of the tendency of many proteins to denature at high temperature (Sun et al., 2002; Taiz and Zeiger, 1998). The expression of HSPs positively correlates with the acquisition of thermotolerance, and the overexpression of HSPs often results in enhanced thermotolerance (Schöffl and Prändl, 1999; Sun et al., 2002). Induction of HSPs is not restricted to heat stress. HSPs are also linked to a number of other abiotic stresses such as drought (Rizhsky et al., 2002) and oxidative stresses (Larkindale and Knight, 2002).

Water makes up more than 70% of the weight of living organisms and is the most important molecule in most life forms. Water stress in its broadest sense includes both drought and salt stress. Conditions of water deficit result in lower plant cell turgor, a decrease in growth rate, limited numbers and area of leaves, growth of roots into deeper moist soil zones, and stomatal closure to reduce evaporation (Taiz and Zeiger, 1998). Acute water deficits impair photosynthesis. Likewise, under high salinity, plants exhibit decreased water uptake and a subsequent reduction in leaf growth rate, which results in restricted photosynthetic capacity (Munns, 2002). The responses of plants to drought and

salt stresses have much in common and involve a number of metabolic and physiological changes, many of which have not been fully characterized.

Plant cells maintain total water potential during drought and salt stress by osmotic adjustment, a process to decrease water potential by accumulation of sugars or other compatible solutes such as proline, glycine betaine, mannitol, and sorbitol. Several transgenic plants which overproduce such solutes have shown some tolerance to drought and salt stress (Chinnusamy et al., 2005). Osmotic adjustment helps to maintain turgor and enables the continuation of cell elongation at lower water potentials. Osmotic adjustment is a mechanism by which plants acclimate to dehydration conditions, like drought and salt stress.

Abscisic acid (ABA) is the major plant hormone involved in the response to salinity, drought, and cold stress (Leung and Giraudat, 1998). The endogenous ABA content increases dramatically in all plant organs under drought and salt stress. Cellular ABA triggers stomatal closure to limit transpirational water loss. ABA also regulates gene expression in response to stress conditions. The main role of ABA in drought and salt stress is to increase tolerance to cellular dehydration and maintain water balance (Zhu, 2002).

Oxidative stress results from the formation of active oxygen species (AOS) that damage or kill cells (Apel and Hirt, 2004). The negative effects of oxidative stress on plants include reduced photosynthesis, leaf injury, shoot and root growth reduction, accelerated senescence, and reduced crop yields. Environmental factors that cause oxidative stress include air pollution such as ozone or sulfur dioxide, oxidant-forming herbicides such as paraquat dichloride, heavy metals, drought, heat and cold stress,

wounding, ultraviolet light, and high intense light that cause photoinhibition (Dat et al., 2000; Mittler, 2002). Oxidative stress also occurs in response to pathogen infection and during senescence (Bolwell, 1999). In many plants, ozone exposure and other oxidative stresses can trigger antioxidant defense system. Increased synthesis of antioxidants and antioxidant enzymes can improve tolerance to oxidative stress.

Several secondary metabolites of plants have been implicated in signaling in response to a variety of abiotic and biotic stresses. Salicylic acid is involved in response to SAR and the hypersensitive response (HR) (Durrant and Dong, 2004; Klessig and Malamy, 1994; Lamb and Dixon, 1997; Shah, 2003). Besides its role in biotic stresses, SA may modulate plant responses to several abiotic stresses. SA levels increase during exposure to ozone or ultraviolet light (Sharma et al., 1996; Yalpani et al., 1994), while pretreatment with SA can protect plants from paraquat-induced oxidative stress (Strobel and Kuc, 1995). Dat et al. (1998) demonstrated the induction of thermotolerance by spraying SA on mustard seedlings. Similarly, wheat (Shakirova et al., 2003) and tomato (Tari et al., 2002) pre-treated with SA exhibited tolerance to salinity stress. In maize, SA reduces the effects of chilling injury (Janda et al., 1999) but increases sensitivity to drought (Nemeth et al., 2002).

There are overlaps in signal transduction between abiotic and biotic stresses. Studies have shown that plants resistant to one stress are often more resistant to others. This phenomenon is known as cross-tolerance (Bowler and Fluhr, 2000; Pastori and Foyer, 2002). For example, ozone treatment triggers induced resistance of Arabidopsis to subsequent infection with *P. syringae* (Sharma et al., 1996). In tomato, salt stress induces accumulation of proteinase inhibitors and activates expression of other wound-related

genes. Salt stress strongly enhances the wounding response both locally and systemically (Dombrowski, 2003). Moreover, cool-season grasses infected with endophytic fungi have enhanced tolerance to drought and mineral stresses (Malinowski and Belesky, 2000).

Application of PGPR increases plant health overall. Precisely how the interaction of plant and PGPR affects the physiology and metabolism in plants is unclear. There is much research concerning the effect of PGPR applied as biocontrol agents on a plant's resistance or tolerance to different plant pathogens. In contrast, only a few PGPR strains have been studied for their capacity to enhance plant tolerance to environmental stresses. Plants with reduced ethylene levels by PGPR with 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity showed a substantial tolerance to flooding stress (Grichko and Glick, 2001b) and metal contaminants (Belimov et al., 2005; Nie et al., 2002). PGPR strain *Paenibacillus polymyxa* can protect *Arabidopsis* against *Erwinia carotovora* (biotic stress) and drought (abiotic stress) (Timmusk and Wagner, 1999). In field experiments, sorghum plants inoculated with *Azospirillum* had higher yield than noninoculated plants under drought stress conditions. *Azospirillum* inoculated sorghum plants had more water content, higher water potential, and lower canopy temperature in their foliage. Hence, they were less drought stressed than noninoculated plants (Sarig et al., 1988). Also, *Azospirillum* spp. have been shown to improve the survival and development of three different species of cactus transplants in the desert soil (Bashan et al., 1999).

II. ELICITATION OF ARABIDOPSIS GROWTH PROMOTION AND INDUCED RESISTANCE BY PGPR

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are free-living rhizobacteria that colonize plant roots and are beneficial (mutualistic) to plants (Glick et al., 1999; Kloepper, 1994). Several rhizobacterial genera have been reported to promote plant growth and yield under greenhouse and/or field conditions. These rhizobacteria include strains of *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Serratia*, and *Streptomyces* (Dobbelaere et al., 2003; Glick et al., 1999; Lucy et al., 2004). The application of PGPR has been widely used in agriculture, horticulture, forestry, and environmental regeneration (Lucy et al., 2004).

Plant growth benefits resulting from PGPR application include increases in germination rate, root and shoot weight, lateral root growth, leaf surface area, chlorophyll content, nitrogen content, and yield. In general, yield is enhanced up to 10% for cereal crops and 15 to 50% for different vegetable crops with PGPR applications (Kloepper, 1994). A growing number of PGPR are being commercialized as biocontrol agents or biofertilizers in the U.S. (Glick et al., 1999; Kloepper et al., 2004b; McSpadden Gardener and Fravel, 2002). In recent years, *Bacillus* spp. have drawn more attention for commercial biofertilizers due to characteristics such as production of heat- and

desiccation-tolerant endospores under stress environments (Emmert and Handelsman, 1999; Handelsman and Stabb, 1996). One of the major challenges for large-scale applications of PGPR products is to maintain high populations of living microorganisms for longer shelf-life. Several commercially available biofertilizer products, such as AgBlend®, BioYield®, and Equity™, include *Bacillus* spp. to facilitate the formulation process.

Many reports on the mode of action of plant growth promotion by PGPR have been published. PGPR can promote plant growth directly through fixation of nitrogen, facilitation of mineral uptake, solubilization of phosphorus, production of siderophores that solubilize and sequester iron, production of phytohormones, or reduction in soil levels of ethylene (Dobbelaere et al., 2003; Glick et al., 1999). Also, PGPR may indirectly promote plant growth by antibiosis against deleterious soil microorganisms, competition for colonization sites or nutrients, or induced systemic resistance (Glick et al., 1999; Handelsman and Stabb, 1996; van Loon et al., 1998).

Several plant-associated bacteria are capable of producing plant growth regulators such as indole-3-acetic acid (IAA), cytokinins, gibberellins, ethylene, or abscisic acid (ABA) (Arshad and Frankenberger, 1998; Costacurta and Vanderleyden, 1995). Some *Bacillus* spp. have been shown to produce IAA (Srinivasan et al., 1996), cytokinins (Timmusk et al., 1999), and gibberellins (Gutierrez Manero et al., 1996; Gutierrez Manero et al., 2001). Ryu et al. (2003a) found volatile compounds released by two PGPR strains, *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a, promoted Arabidopsis growth in a closed I-plate condition. The volatile compounds, 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, were identified in both *Bacillus* strains. Exposure to

2,3-butanediol vapors stimulated Arabidopsis growth in a dose-dependent manner. Further, *B. subtilis* mutant strains defective in the production of acetoin and 2,3-butanediol were unable to promote plant growth.

As indicated in the examples above, most studies on mechanisms have emphasized bacterial determinants of the growth promotion response. In most cases, stimulation of plant growth has been reported by measurements of total biomass or yield. In contrast, there is much less information regarding the effects on plant morphology and physiology (e.g., root weight, root length). In addition, little is known about how plant development and growth rate are affected by PGPR, and whether PGPR can still enhance plant growth on older, more developed plants. In order to understand these growth effects of PGPR on the plant, Arabidopsis was used as a model system. The advantages of using Arabidopsis include small plant size, small genome size, rapid life cycle, and extensive literature defining many aspects of plant morphogenesis and physiology related to growth and development under experimental conditions (Meinke et al., 1998). The specific objectives of this study were (1) to determine if PGPR will advance the developmental stage of Arabidopsis by increasing the rate of growth and (2) to determine if PGPR can elicit growth promotion and induced systemic resistance on Arabidopsis at different growth stages.

MATERIALS AND METHODS

PGPR strains and inoculum preparation.

Seven PGPR strains were used in this study: *Pseudomonas fluorescens* 89B-61, *Serratia marcescens* 90-166, *Bacillus pasteurii* C9, *B. subtilis* GB03, *B.*

amyloliquefaciens IN937a, *B. pumilus* SE34, and *B. pumilus* T4. All PGPR strains were maintained in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) amended with 20% glycerol at –80°C. PGPR inoculum was prepared by harvesting bacterial cells from 24 h cultures on tryptic soy agar (TSA) plates at 28°C. The inoculum was suspended in sterile distilled water to yield 10^8 colony forming units (cfu) per ml.

Plant material and growth conditions.

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were imbibed in 0.15% agar and cold-treated at 4°C for 48 to 72 hours before seeding. Seeds were then planted on the top of Speedling soilless mix (Speedling Inc., Sun City, FL) and maintained in greenhouse conditions with natural light at 22 to 25°C and 70 to 80% relative humidity.

PGPR effect on Arabidopsis growth stage development and growth rate.

Arabidopsis seeds were sown into 70 x 35 cm Styrofoam flats (Speedling Inc., Sun City, FL), each containing 128 seeding wells (2.5 x 2.5 cm each), and maintained in the greenhouse. Strain GB03 was applied at $10^8 – 10^9$ cfu/ml immediately after seeding. The definition of different Arabidopsis growth stages was modified from Boyes et al. (2001), so that leaf development stages were mainly used to analyze Arabidopsis growth. With this system, the first growth stage is when the cotyledons are fully open. Subsequent growth stages are numbered according to the number of rosette leaves that were larger than 1 mm in length. The final growth stage (flowering stage) occurs when the inflorescence has emerged. In our experiments, we determined the number of days required to attain each growth stage. Eighty plants per treatment were used. For both

GB03-treated and nontreated Arabidopsis growth rates, total leaf surface area, rosette leaf diameter, and leaf number were measured at 7, 14, and 21 days after seeding. Twenty plants per treatment were used. The entire experiment was conducted two times.

PGPR effect on elicitation of growth promotion and induced systemic resistance in different Arabidopsis growth stages.

Seven PGPR strains, 89B-61, 90-166, C9, GB03, IN937a, SE34, and T4, were used in this study. For seed treatment, 10 to 15 Arabidopsis seeds were mixed with 50 ml of each PGPR strain suspension (10^8 cfu/ml), fertilizer (10-20-10), 3 mM benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester (BTH, Actigard) (Syngeta, Research Triangle Park, NC, USA), or sterile distilled water prior to seeding. After gently mixing for 1 minute, each seed treatment was poured into a 10 x 10 cm pot with Speedling soilless mix (Speedling Inc., Sun City, FL). Ten replications per treatment were used. The experiment was conducted three times.

For determining if PGPR elicit growth promotion on plants at different growth stages, 50 ml of the same 10 treatments described above were used to drench each pot at the following days after seeding: day-0 (seed), day-7 (2-leaf stage), day-14 (3-leaf stage), and day-21 (7-leaf stage). The experiment was a 10 (7 PGPR strains, 2 chemicals, and a water control) x 4 (different growth stage application times) factorial with randomized complete block (RCB) design. Ten replications per treatment were used. The experiment was conducted three times.

Effect on plant growth was assessed by measuring leaf weight, root weight, total fresh weight, and total leaf surface area (TLSA) by an integrated digital video image

analysis system, AGVISION system (AgImage Plus Version 1.08, Decagon Devices, Inc., and Panasonic CCTV camera Model WV-BL200, Pullman, WA, USA), at 28 days after seeding.

For the disease resistance assay, the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000, suspended at 10^6–10^7 cfu/ml in sterile water and amended with 200 μg/L Tween 20 (Sigma, St. Louis, MO, USA), was sprayed onto the leaves of 28-day-old Arabidopsis of all treatments until run off. Inoculated plants were placed into a dew chamber (100% humidity), kept in the dark for two days, and then transferred to greenhouse for continuing disease development. Seven days after pathogen challenge, disease severity was measured by a disease index ranging from 0 to 10 (0 = healthy; 1 = 1-10 % of symptomatic leaf area per plant; 2 = 11-20 % of symptomatic leaf area per plant; 3 = 21-30 % of symptomatic leaf area per plant; 4 = 31-40 % of symptomatic leaf area per plant; 5 = 41-50 % of symptomatic leaf area per plant; 6 = 51-60 % of symptomatic leaf area per plant; 7 = 61-70 % of symptomatic leaf area per plant; 8 = 71-80 % of symptomatic leaf area per plant; 9 = 81-90 % of symptomatic leaf area per plant; 10 = 91-100 % of symptomatic leaf area per plant).

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using JMP software (SAS Institute, Cary, NC). Treatment means were separated by Fisher's protected least significant difference (LSD) test at $P = 0.05$.

RESULTS

Effect of PGPR on Arabidopsis growth stage development and growth rate.

Bacillus strain GB03 has been shown to elicit growth promotion in several crop plants, as well as in Arabidopsis (Kloepper et al., 2004b; Ryu et al., 2003a). Our results showed that *Bacillus* strain GB03 significantly shortened the time to attain Arabidopsis growth stages between the three-leaf to ten-leaf stages and caused significantly earlier inflorescence development than the water control (Figure 1). There was no significant difference between GB03-treated and nontreated Arabidopsis in the time to attain growth stage one (cotyledons fully open) or the two-leaf stage. *Bacillus* strain GB03 boosted the rate of Arabidopsis development between 7 to 12 days after seeding (Figure 1).

At 7, 14, and 21 days after seeding, 20 plants of each GB03-treated Arabidopsis and nontreated control were measured for total leaf surface area, rosette leaf diameter, and leaf number to obtain the growth rate. The growth rate for GB03-treated Arabidopsis was best described by the model Y (total leaf surface area) = –4.3352 + 0.7828 X (days after seeding) ($R^2 = 0.67$, $P < 0.0001$) (Figure 2A), Y (rosette diameter) = –0.3061 + 0.3752 X ($R^2 = 0.73$, $P < 0.0001$) (Figure 2B), and Y (leaf number) = 0.2185 + 0.2272 X ($R^2 = 0.69$, $P < 0.0001$) (Figure 2C). The growth rate for nontreated Arabidopsis was best described by the model Y (total leaf surface area) = –0.4783 + 0.2069 X ($R^2 = 0.61$, $P < 0.0001$) (Figure 2A), Y (rosette diameter) = 0.2633 + 0.1878 X ($R^2 = 0.77$, $P < 0.0001$) (Figure 2B), and Y (leaf number) = 1.4711 + 0.0567 X ($R^2 = 0.23$, $P < 0.0001$) (Figure 2C). Regression models showed that *Bacillus* strain GB03 had higher growth rates, based on total leaf surface area, rosette diameter, and leaf number, compared to the nontreated control (Figure 2). At 7 days after seeding, GB03 had significantly higher total leaf surface area and rosette diameter but did not show significant difference on leaf number

compared to nontreated control. At both 14 and 21 days after seeding, GB03 significantly increased Arabidopsis growth on all three growth parameters.

Effect of PGPR applied as seed treatment on Arabidopsis growth.

The effect of PGPR applied as seed treatment on total leaf surface area, total fresh weight, and leaf weight was measured at 28 days after seeding (Table 1). Seed treatment of *Bacillus* strain GB03 significantly increased total leaf surface, total fresh weight, and leaf weight. Two *Bacillus* strains, C9 and SE34, significantly increased leaf weight. Two other *Bacillus* strains, IN937a and T4, and *Serratia* strain 90-166 had no significant effect on plant growth when they were applied as seed treatment. However, *Pseudomonas* strain 89B-61 significantly reduced total leaf surface area and leaf weight. BTH, a chemical inducer for systemic acquired resistance, significantly reduced total leaf surface, total fresh weight, and leaf weight. Seed treatment with fertilizer had no effect on plant growth.

Effect of PGPR on elicitation of growth promotion at different Arabidopsis growth stages.

Different PGPR and chemical treatments were applied at four different growth stages: day-0 (seed), day-7 (2-leaf stage), day-14 (3-leaf stage), and day-21 (7-leaf stage). Total foliar fresh weight was measured at 28 days after seeding (Table 2). All seven PGPR strains significantly increased total foliar fresh weight when applied at 0 and 7 days after seeding. When applied at 14 days and 21 days after seeding, *Bacillus* strains SE34 and T4 were still able to significantly increase total foliar fresh weight. Two

Gram-negative strains, 89B-61 and 90-166, significantly increased total foliar fresh weight when they were applied at 21 days after seeding. Fertilizer significantly increased growth when applied at all four growth stages. However, BTH-treated Arabidopsis had the lowest total foliar fresh weight at all four application times (Table 2).

Bacillus strains SE34 and T4 had the greatest growth promotion compared to other *Bacillus* strains used in this study. Both strains significantly enhanced Arabidopsis growth at all four growth stages. Reanalyzed data showed that both strains enhanced total foliar fresh weight the most when they were applied at seeding compared to 21 days after seeding (Figure 3).

Effect of PGPR on induced systemic resistance against *Pseudomonas syringae* pv. tomato at different growth stages of Arabidopsis.

All PGPR strains elicited significant disease protection against *Pseudomonas syringae* pv. tomato at some growth stage of Arabidopsis (Table 3). Seed treatment with *Serratia* strain 90-166 and *Bacillus* strain T4 significantly reduced disease severity compared to the water control. *Pseudomonas* strain 89B-61, *Serratia* strain 90-166, and BTH elicited disease protection at all four growth stages. *Bacillus* strain C9 and GB03 only elicited disease protection at the most advanced growth stage. Application of fertilizer did not result in any disease protection.

DISCUSSION

Our results showed that *Bacillus* strain GB03 enhanced plant development as indicated by decreasing the time required for treated Arabidopsis to reach each growth

stage from the three-leaf stage to inflorescence emergence (Figure 1). GB03-treated plants typically reached a given growth stage 2 days earlier than nontreated control plants. In addition to enhancing plant development, GB03 also elicited growth promotion as indicated by enhanced weights of leaves and flowers 40 days after seeding. This finding confirms a previous report (Ryu et al., 2003a) showing that *Bacillus* strain GB03 elicited growth promotion on Arabidopsis.

The finding that *Bacillus* strain GB03 enhanced plant development suggested that the bacterium elicited an increase in the growth rate of Arabidopsis. Support for this suggestion was obtained when we measured three growth parameters, total leaf surface area, rosette leaf diameter, and leaf number over time. Regression models showed that GB03-treated Arabidopsis had higher growth rates on all three growth parameters (Figure 2). However, GB03 did not elicit enhanced leaf number at 7 days after seeding. This result is consistent with previous experiments that showed the days to attain the two-leaf stage (around 7 days after seeding) were not significantly different with GB03 from the nontreated control (Figure 1). Our findings showed that PGPR not only resulted in a larger sized plant, but also boosted the rate of plant growth. While many reports demonstrate that PGPR enhance growth at one or two times during the season, we could not find any reports that specifically examined the effect of PGPR on the rate of plant growth, and hence, the finding here that PGPR increase the rate of Arabidopsis growth enhances our understanding of mechanisms and effects of PGPR.

Seed treatment and soil drench are two common methods used for PGPR application. When PGPR were applied as seed treatment, only *Bacillus* strain GB03 significantly increased Arabidopsis growth in all three growth parameters (Table 1). In

some cases, some PGPR strains had negative impacts on Arabidopsis germination rate and growth (data not shown). When we attempted to inoculate Arabidopsis seeds with PGPR suspension in the sterilized microtiter plate system, many Arabidopsis seeds did not survive from the high dose of inoculum (10^8 cfu/ml) (data not shown). In conclusion, growth promotion by seed treatment with PGPR is strain-specific. It is likely that high concentrations of PGPR negatively affect Arabidopsis before seed germination, probably due to the thinner seed coat of Arabidopsis compared to other crop plants.

Different PGPR and chemical treatments were applied at four different growth stages, seed (day-0), 2-leaf stage (day-7), 3-leaf stage (day-14), and 7-leaf stage (day-21). PGPR significantly enhanced Arabidopsis growth when applied at the seed and 2-leaf stages (Table 2 and Figure 3). Therefore, elicitation of Arabidopsis growth promotion by PGPR was best between 0 and 7 days. Two *Bacillus* strains, SE34 and T4, were the best strains for Arabidopsis growth promotion in this study. These two strains elicited growth promotion at all four growth stages, as did fertilizer; moreover, they elicited greater growth promotion compared to fertilizer when they were applied at seeding (day-0) (Table 2). In other repeated experiments, the performance of PGPR varied by strain and by application time, but the same pattern emerged (data not shown).

There are several reports of the negative effect on the plant growth by BTH under no or low disease pressure (Heil and Baldwin, 2002; Heil et al., 2000). BTH (Actigard) is a commercial product which is registered as a plant activator for systemic acquired resistance. BTH has been shown to be a consistent and effective chemical to activate plant resistance against several plant diseases in the field (Oostendorp et al., 2001). Therefore, BTH was used in this experiment for the comparison with PGPR strains on the

growth promotion and induced resistance. Our results showed that BTH treatment resulted in significantly less plant growth compared to PGPR at all four growth stages (Table 2), confirming reports on other plant species. Also, reanalyzed data showed that BTH significantly reduced Arabidopsis growth at all four growth stages compared to the water control (data not shown). Although BTH had the strongest disease protection (Table 3), it had a negative impact on the plant growth.

Elicitation of induced systemic resistance against the bacterial pathogen *Pseudomonas syringae* pv. tomato by PGPR was dependent on PGPR strains and application times. Our results showed that two Gram-negative PGPR strains had better and longer induced systemic resistance compared to *Bacillus* spp. (Table 3). On the other hand, *Bacillus* spp. reduced disease severity differentially by strains. Some *Bacillus* strains had shorter duration time of induced systemic resistance; hence they only induced plant resistance one week before pathogen challenge (Table 3).

The relationship between plant growth promotion and induced resistance by PGPR was previously investigated in tobacco against blue mold (*Peronospora tabacina*) (Zhang et al., 2004). In this study, *Serratia* strain 90-166 showed a positive relationship between growth promotion and disease reduction. However, our results showed that strain 90-166 can still induce disease resistance without a significant growth promotion when applied at older development stages (day-14 and 21) of Arabidopsis (Table 2 and 3). Therefore, the relationship between plant growth promotion and induced resistance may be very complex and affected by several different factors such as plant species, PGPR strains, and environmental factors.

In conclusion, *Bacillus* strain GB03 significantly enhanced Arabidopsis growth rate and advanced its development stage. The most effective time to elicit Arabidopsis growth promotion by PGPR is at the early stages of development . However, induced systemic resistance by PGPR may vary according to plant species and PGPR strains.

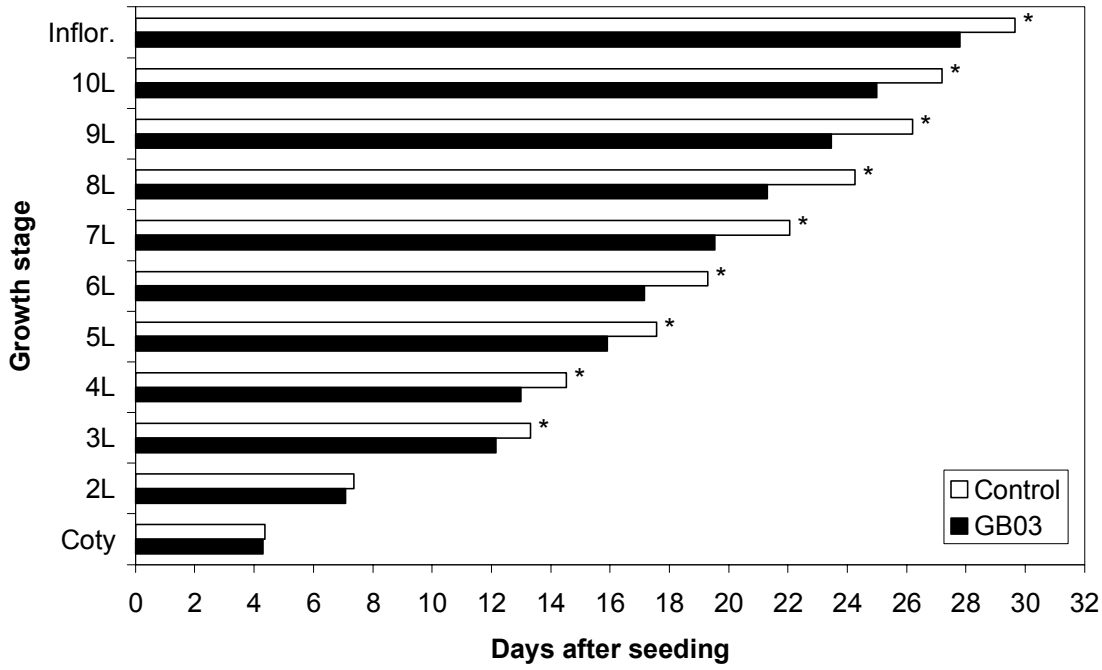
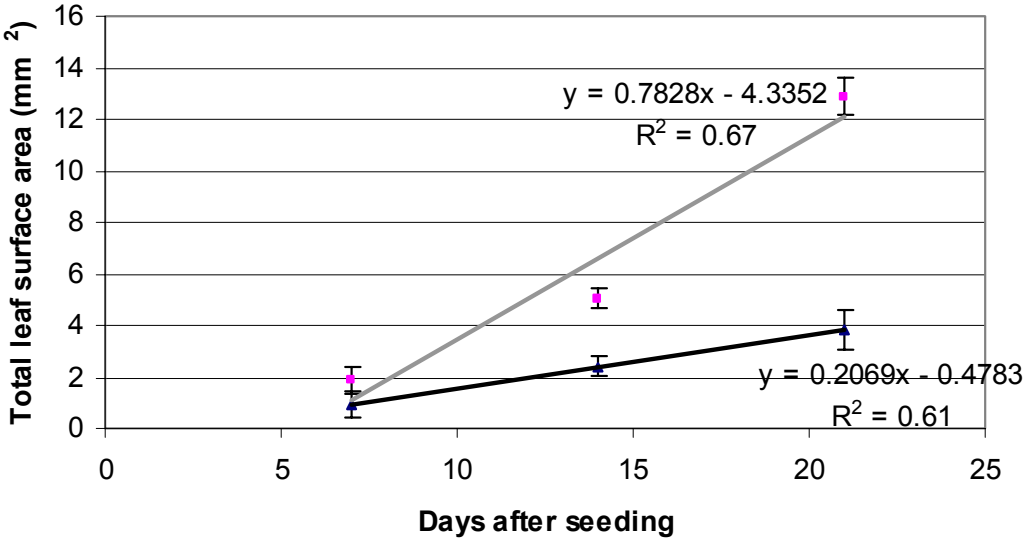
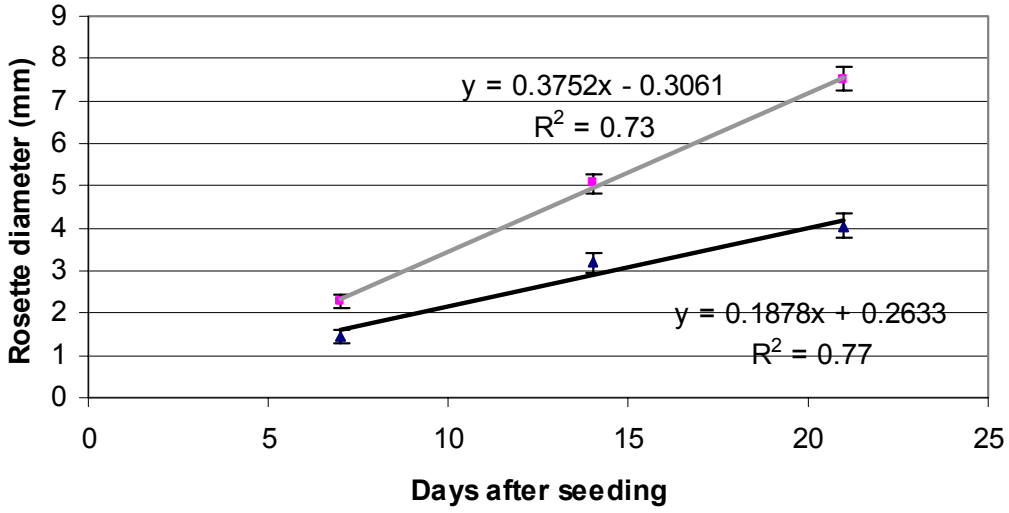


Figure 1. Reduced time required for Arabidopsis to reach various growth stages following treatment with *Bacillus* strain GB03. The definition of different Arabidopsis growth stages was modified from Boyes et al. (2001). The first stage is cotyledons fully opened (Coty), then different stages according to the number of rosette leaves that were larger than 1 mm in length (2L to 10L), and finally inflorescence emergence (Inflor.). Days required to attain each development stage were recorded. Values are the means of 80 plants. * Indicates values that are significantly different using Fisher's LSD test at $P = 0.05$.

(A)


Total leaf surface area (mm 2)
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
Days after seeding
y = 0.7828x - 4.3352
R2 = 0.67
y = 0.2069x - 0.4783
R2 = 0.61

(B)


Rosette diameter (mm)
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
Days after seeding
y = 0.3752x - 0.3061
R2 = 0.73
y = 0.1878x + 0.2633
R2 = 0.77

(C)

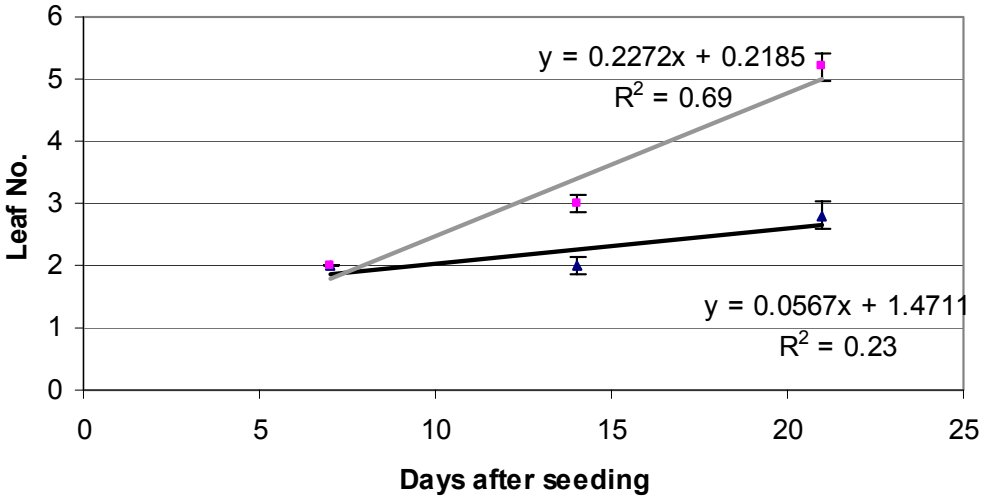


Figure 2. Elicitation by *Bacillus* strain GB03 of enhanced rate of Arabidopsis growth. (A) Total leaf surface area. (B) Rosette diameter. (C) Leaf number. Rectangle marker (■) represents GB03 treated Arabidopsis and triangle marker (▲) represents nontreated control. Gray line indicates the regression line for GB03-treated Arabidopsis and black line indicates the regression line for nontreated control. Values are the means of 20 plants. Standard error (工) uses a pooled estimate of error variance.

Table 1. Effect of PGPR on 28-day-old Arabidopsis total leaf surface area, total fresh weight and leaf weight when they were applied as seed treatment.

Treatment	Total leaf surface area (cm^2)	Total fresh weight (mg)	Leaf weight (mg)
Control	3.92^{bc}	142.89^{bc}	101.56^{c}
89B-61	2.35^{d}	96.44^{cd}	56.89^{d}
90-166	3.94^{bc}	141.40^{bcd}	104.00^{bc}
C9	4.98^{ab}	171.25^{ab}	146.00^{a}
GB03	5.41^{a}	203.00^{a}	147.00^{a}
IN937a	3.24^{cd}	113.50^{cd}	83.10^{cd}
SE34	4.75^{ab}	182.67^{ab}	142.83^{ab}
T4	3.95^{bc}	128.00^{bcd}	105.80^{bc}
Fertilizer	2.68^{cd}	114.70^{cd}	71.10^{cd}
BTH	2.36^{d}	87.22^{d}	59.89^{d}
LSD ($P = 0.05$)	1.32	55.59	38.85

Values are means of 10 replications. Superscripted letters indicate values within the same column that are either statistically significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$.

Table 2. Effect of PGPR on 28-day-old Arabidopsis total foliar fresh weight when they were applied at day-0 (seed), day-7 (2-leaf stage), day-14 (3-leaf stage), and day-21 (7-leaf stage).

Treatment	Day-0	Day-7	Day-14	Day-21
Control	47.67^{e}	45.67^{e}	51.20^{de}	50.80^{de}
89B-61	159.00^{ab}	112.33^{bcd}	84.90^{cd}	134.20^{ab}
90-166	193.10^{a}	131.90^{bc}	86.60^{bcd}	97.10^{bc}
C9	140.20^{bc}	105.00^{cd}	90.00^{bcd}	83.30^{cd}
GB03	98.70^{d}	103.40^{cd}	73.10^{cde}	84.80^{cd}
IN937a	107.50^{cd}	87.70^{d}	87.50^{bcd}	81.30^{cd}
SE34	172.90^{ab}	111.00^{bcd}	125.00^{b}	107.80^{bc}
T4	167.11^{ab}	141.20^{ab}	93.50^{bc}	105.00^{bc}
Fertilizer	146.70^{b}	170.50^{a}	198.80^{a}	150.89^{a}
BTH	24.00^{e}	31.30^{e}	37.10^{e}	34.20^{e}
LSD (P = 0.05)	35.48	35.35	40.07	38.50

Values are means of 10 replications. Superscripted letters indicate values within the same column that are either statistically significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$.

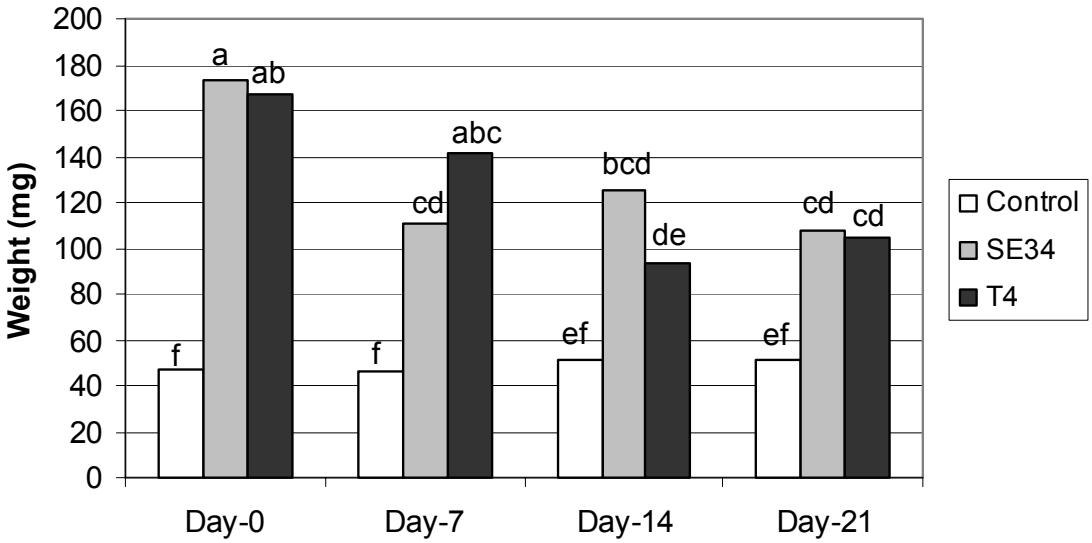


Figure 3. Effect of *Bacillus* strains SE34 and T4 applied at different Arabidopsis growth stages on total foliar fresh weight of 28-day-old Arabidopsis. Values are the means of 10 replications (LSD = 43.36). Different letters indicate significant differences using Fisher's LSD test at $P = 0.05$.

Table 3. Elicitation of induced disease resistance against *Pseudomonas syringae* pv. tomato by PGPR at different growth stages of Arabidopsis[a].

Treatment	Disease Index (0 -10)*				
	Seed treatment	Day-0	Day-7	Day-14	Day-21
Control	5.43^{abc}	7.67^{a}	6.75^{a}	6.50^{a}	6.25^{ab}
89B-61	4.67^{bc}	3.50^{de}	4.00^{cd}	3.14^{e}	3.00^{f}
90-166	3.33^{de}	4.25^{d}	3.75^{d}	5.17^{bc}	4.18^{de}
C9	4.78^{bc}	7.86^{a}	6.45^{a}	6.33^{a}	3.11^{f}
GB03	4.50^{cd}	6.86^{ab}	5.57^{ab}	6.14^{ab}	3.89^{ef}
IN937a	4.17^{cde}	5.50^{c}	6.67^{a}	2.71^{e}	5.00^{cd}
SE34	5.86^{ab}	5.91^{bc}	4.33^{cd}	6.67^{a}	3.40^{ef}
T4	3.14^{e}	6.13^{bc}	5.00^{bc}	4.50^{cd}	5.71^{bc}
Fertilizer	6.14^{a}	6.86^{ab}	5.82^{ab}	6.75^{a}	6.88^{a}
BTH	6.14^{a}	2.67^{e}	3.67^{d}	3.55^{de}	3.50^{ef}
LSD (P = 0.05)	1.29	1.21	1.18	0.98	0.95

[a] Bacterial pathogen suspension (10^6–10^7 cfu/ml) was sprayed onto leaves of 28-day-old Arabidopsis. Disease severity was recorded seven days after pathogen challenge. Values are means of 10 replications. Superscripted letters indicate values within the same column that are either statistically significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at P = 0.05.

* Disease index was measured from 0 – 10 (0 = healthy; 10 = most severely diseased).

III. EFFECT OF PGPR ON ARABIDOPSIS ROOT MORPHOLOGY

INTRODUCTION

Healthy root systems are vital to plant growth and survival. Water and nutrients are absorbed and translocated from the soil by the root system. Root hairs greatly increase root surface area and diameter, and thus they are generally considered to assist plants in nutrient acquisition, anchorage, and microbe interaction (Grierson and Schiefelbein, 2002). Plant growth-promoting rhizobacteria (PGPR) colonize plant roots and can stimulate plant growth and reduce several plant diseases (Handelsman and Stabb, 1996; Kloepper, 1994). Application of PGPR in agriculture can increase crop yields, reduce the need for chemical fertilizers, and contribute to sustainable agricultural production (Cook, 2000; Lucy et al., 2004).

Plant growth promotion by PGPR involves various mechanisms that are still not fully understood. Various strains of PGPR can promote plant growth directly through nitrogen fixation, siderophore production, facilitation of mineral uptake, solubilization of phosphorus, production of phytohormones such as indole-3-acetic acid (IAA), or by lowering soil levels of ethylene (Dobbelaere et al., 2003; Glick et al., 1999). Many studies have been published showing that inoculation of PGPR can significantly increase root and shoot growth. PGPR interact directly with plant root systems and have a positive

impact on root biomass, morphology, and physiology (Cleyet-Marel et al., 2001; Fallik et al., 1994).

The association between *Azospirillum* spp. and the plant root has been extensively investigated (Okon, 1994) and *A. brasilense* strain Cd has been shown to alter root morphology. Inoculation of wheat with Cd enhanced cell division in the root tips and increased the size of the elongation zone (Levanony and Bashan, 1989). Significant enhancement in root dry weight by 50% and root length by 35% were observed in tomato seedlings inoculated with *A. brasilense* Cd at a concentration of 5×10^8 cfu/ml (Hadas and Okon, 1987). Numbers of tomato root hairs were significantly enhanced by inoculation with 1×10^8 cfu/ml. At a higher inoculum concentration (5×10^8 and 1×10^9 cfu/ml), the root elongation zone was inhibited, but a high density of root hairs occurred (Hadas and Okon, 1987). Root hairs of Arabidopsis inoculated with *A. brasilense* Sp-245 were at least twice as long as those of the noninoculated control at the same physiological development stages (Dubrovsky et al., 1994).

In addition to *Azospirillum* spp., several different taxa of PGPR have been shown to induce morphological modification in roots. *Phyllobacterium* sp. strain 29-15 significantly promoted lateral root length of oilseed-rape (*Brassica napus*) by increasing both the density and the elongation rate of lateral roots (Larcher et al., 2003). *Pseudomonas fluorescens* strain A6RI was shown to increase root length, surface area, and volume in tomato (Gamalero et al., 2002). A nonfluorescent *Pseudomonas* strain, PsJN, increased the number of lateral roots and root hairs in tomato but led to shorter root systems (Pillay and Nowak, 1997). Various PGPR strains containing

1-aminocyclopropane-1-carboxylate (ACC) deaminase, such as *P. putida* Am2, *P. putida* Bm3, *Alcaligenes xylosoxidans* Cm4, and *Pseudomonas* sp. Dp2, significantly increased root elongation of spring rape (*Brassica napus* var. *oleifera* L.) under phosphorus-sufficient conditions; whereas root elongation under phosphorus-deficient conditions was not affected or was inhibited by the bacteria. Also, bacterial inoculations significantly decreased the length and numbers of root hairs around root tips at both levels of phosphate (Belimov et al., 2002). Interestingly, *P. thivervalensis* strain MLG45, which naturally colonized Arabidopsis roots, reduced the root length in a dose-dependent manner (Persello-Cartieaux et al., 2001).

Arabidopsis thaliana has been shown to be an ideal model system for studies of plant-microbe interactions and has been the primary plant used to elucidate signaling pathways of SAR and ISR (Glazebrook et al., 1997; Pieterse et al., 1998; Thomma et al., 2001). The advantages of using Arabidopsis include its compact size, small genome size, and rapid life cycle coupled with the extensive literature defining many aspects of its morphogenesis and physiology (Meinke et al., 1998). In this study, we selected Arabidopsis as a model to understand effects of PGPR on plant root systems. The specific objectives were to develop a simple and nondestructive system for observing the effects of PGPR on Arabidopsis root systems and to observe root and root hair growth in relation to inoculation with PGPR strains.

MATERIALS AND METHODS

PGPR strains and inoculum preparation.

Eight different PGPR strains were used in this study: *Pseudomonas fluorescens* 89B-61, *Serratia marcescens* 90-166, *Bacillus pasteurii* C9, *B. subtilis* GB03, *B. amyloliquefaciens* IN937a, *B. pumilus* INR7, *B. pumilus* SE34, and *B. pumilus* T4. All PGPR strains were maintained in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) amended with 20% glycerol at –80°C. PGPR inoculum was prepared by harvesting bacterial cells from 24h cultures on tryptic soy agar (TSA) plates at 28°C.

Production of ACC deaminase by PGPR.

Because production of ACC deaminase has previously been associated with morphological effects of PGPR on roots, a test was conducted to determine if PGPR strains produced this enzyme to utilize ACC, an ethylene precursor. Seven PGPR strains, 89B-61, 90-166, GB03, IN937a, INR7, SE34, and T4, were grown in liquid DF salt medium (one liter medium contains 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2 g glucose, 2 g gluconic acid, 2 g citric acid, 100 mg $FeSO_4 \cdot 7H_2O$, 10 µg H_3BO_3, 11.19 µg $MnSO_4 \cdot H_2O$, 124.6 µg $ZnSO_4 \cdot 7H_2O$, 78.22 µg $CuSO_4 \cdot 5H_2O$, 10 µg MoO_3, pH 7.2) with 2 g $(NH_4)_2SO_4$ or 3 mM ACC (1-aminocyclopropane-1-carboxylic acid) (Sigma) as nitrogen source at room temperature for 72 hours at 200 rpm. ACC solution was filter-sterilized before being added into sterilized DF salt medium. Utilization of ACC by PGPR was determined if ACC containing DF salt medium turned turbid which is considered an indication of the present of ACC deaminase (Glick et al., 1999; Penrose and Glick, 2003).

Plant material and growth conditions for root morphology experiment.

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized with 95% alcohol for 5 min, then 10% Clorox with 0.1% Triton X-100 for another 5 min, and washed five times in sterile water. Sterilized seeds were suspended in 0.15% agar and cold-treated at 4°C for 48 to 72 hours before seeding.

For the root morphology experiment, Arabidopsis seeds were placed in the plant medium (2.5 mM 2-(N-Morpholino) ethanesulfonic acid (MES), 2 mM KNO_3, 1 mM KH_2PO_4, 0.5 mM $CaSO_4$, 0.5 mM $MgCl_2$, 50 μM NaFe-EDTA, 6 μM $MnSO_4 \cdot H_2O$, 4 μM H_3BO_3, 1 μM $CuSO_4 \cdot 5H_2O$, 0.9 μM $ZnSO_4 \cdot 7H_2O$, 0.1 μM $Na_2MoO_4 \cdot 2H_2O$, pH 5.7, adjusted with KOH) with 1.5% agargel (Sigma) in 9 x 9 cm Petri dishes. With PGPR treatments, bacteria were suspended in 50°C plant medium with 1.5% agargel to yield 10^6-10^9 colony forming units (cfu) per ml. This suspension was then poured into Petri dishes. In each plate, five Arabidopsis seeds were placed on the solidified medium at a distance of 1.5 cm from each other and from the plate edges. The Petri dishes were placed vertically in a growth chamber at 23°C and 16h/8h light/dark photoperiod. Six replications per treatment were used. Each replication contains five seedlings. The experiment was conducted three times. Total root length was measured at 4 and 7 days after seeding. Photos were taken at 14 days after seeding with a digital camera (OLYMPUS Camedia 3000, USA).

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using JMP software (SAS Institute, Cary, NC). Treatment means were separated by Fisher's protected least significant difference (LSD) test at $P = 0.05$.

RESULTS

Production of ACC deaminase by PGPR.

Results showed that five *Bacillus* strains, GB03, IN937a, INR7, SE34, and T4, and *Serratia* strain 90-166 did not grow in ACC containing DF medium, and hence, they did not produce ACC deaminase. However, *Pseudomonas* strain 89B-61 grew in ACC-containing medium, suggesting that this strain utilized ACC as its nitrogen source by producing ACC deaminase. All PGPR strains tested in this study grew in DF medium containing $(NH_4)_2SO_4$ as their nitrogen source.

PGPR effect on Arabidopsis root morphology.

Eight PGPR strains and four inoculum concentrations, 10^6 to 10^9 cfu/ml, were used to study the effect on Arabidopsis root morphology. At 10^6 cfu/ml, three *Bacillus* strains, GB03, IN937a, and INR7, significantly reduced Arabidopsis total root length 4 days after seeding (Table 1). At 10^7 cfu/ml, five *Bacillus* strains significantly reduced total root length (Table 1). However, at the same concentration, *Serratia* strain 90-166 significantly enhanced total root length. At 10^8 cfu/ml, five *Bacillus* strains and *Pseudomonas* strain 89B-61 significantly reduced total root length. Only *Bacillus* strain IN937a did not induce sufficient plant root growth to measure. When the concentration was 10^9 cfu/ml, *Bacillus*-treated Arabidopsis did not survive. *Pseudomonas* strain 89B-61 and *Serratia* strain 90-166 significantly reduced root length at 10^9 cfu/ml.

At 7 days after seeding with 10^6 cfu/ml, five *Bacillus* strains and *Serratia* strain 90-166 significantly reduced total root length (Table 2). At 10^7 cfu/ml, all six *Bacillus*

strains significantly reduced total root length. However, at the same concentration, *Pseudomonas* strain 89B-61 significantly enhanced total root length. At 10^8 cfu/ml, all PGPR strains, except INR7, significantly reduced total root length compared to the water control. *Bacillus* strain INR7 did not survive at 7 days after seeding. However, at the same concentration, two Gram-negative strains, 89B-61 and 90-166, had significantly higher total root length compared to all *Bacillus* strains (Table 2).

PGPR effect on Arabidopsis root hair development.

At 14 days after seeding, *Bacillus*-treated Arabidopsis had a noticeable effect on root hair development (Figure 1 and 2). Arabidopsis root hair density and length were greater when the inoculum concentration of *Bacillus* strains was higher (Figure 2).

DISCUSSION

Several strains of PGPR that produce ACC deaminase have been shown to increase root elongation, improve seedling survival, and enhance stress tolerance (Glick et al., 1998; Grichko and Glick, 2001a). Glick et al. (1998) proposed that root elongation results when ACC deaminase produced by such strains break down ethylene, which inhibits root elongation. Our results with *Pseudomonas* strain 89B-61 support this model, as this strain produced ACC deaminase. However, none of the *Bacillus* strains produced ACC deaminase. Therefore, some unknown mechanism that affects root morphology is operable with these strains.

At 10^7 cfu/ml, two Gram-negative PGPR strains, 89B-61 and 90-166, elicited the greatest enhancement of total root length compared to other inoculum concentrations

(Table 1 and 2). These two strains enhanced the length of lateral roots, while the primary root length and the number of lateral roots were not significantly different from the water control (data not shown). In some cases, the primary root and lateral roots are indistinguishable; hence, the measurement of total root length was used throughout this study. These results support the finding that *Phyllobacterium* strain 29-15, a Gram-negative PGPR, did not increase the primary root length of *Brassica napus* but significantly enhanced both lateral root density and lateral root length (Larcher et al., 2003).

Several reports have shown that at a higher inoculum concentration (5×10^8 and 1×10^9 cfu/ml for instance), Gram-negative PGPR, such as *Azospirillum* and *Pseudomonas* spp., significantly reduced plant root length (Hadas and Okon, 1987; Persello-Cartieaux et al., 2001; Pillay and Nowak, 1997). Our findings that our two Gram-negative PGPR strains significantly reduced Arabidopsis total root length at 10^9 cfu/ml agree with these previous reports.

In comparison to Gram-negative bacteria, there are few reports on how *Bacillus* spp. affect plant root development. Whipker et al. (2001) reported that the commercial product BioYield™, which includes *B. amyloliquefaciens* strain GB99 and *B. subtilis* strain GB03, had a negative effect on ornamental cuttings' root growth when applied at rates higher than 1:20 (product : volume of potting medium). Our finding that higher concentrations of *Bacillus* strains reduced Arabidopsis total root length (Table 1, 2 and Figure 1) support the results of Whipker et al. Moreover, Arabidopsis did not survive at the highest (10^9 cfu/ml) inoculum concentration. However, when we inoculated *Bacillus* spp. strains at 10^9 cfu/ml under greenhouse conditions, Arabidopsis not only survived,

but its leaf weight was significantly higher than the water control (data not shown). In potting system, regular watering washed away non-colonized PGPR and reduced the population of free-living PGPR, which may compete for nutrients for their growth. Yet in Petri dish system, high populations of PGPR remained in the same plate and competed for nutrients with plants. Therefore, in Petri dish system lower PGPR inoculum should be used to avoid the negative effect.

Using Arabidopsis as a model system, Dubrovsky et al. (1994) showed that *Azospirillum brasilense* Sp-245 significantly increased root length only in plant medium containing 1% sucrose compared to no sucrose medium. However, the root hair growth was similar in both 1% sucrose and no sucrose medium. The plant medium used in our study did not contain any carbon source to support the growth of PGPR, which usually outgrow Arabidopsis. Although *Bacillus* spp. PGPR strains reduced Arabidopsis total root length, they enhanced root hair density and root hair length (Figure 2). Moreover, *Bacillus* strains affected Arabidopsis root and root hair growth in a dose-dependent manner.

The phenomenon of root reduction and root hair stimulation elicited by our *Bacillus* spp. PGPR strains is similar to "cytokinin root syndrome" (Su and Howell, 1992). Exogenous cytokinin inhibited Arabidopsis primary root elongation, but stimulated root hair elongation. *Paenibacillus polymyxa*, a close species to *Bacillus*, produced cytokinin (Timmusk et al., 1999) as one of the growth promotion mechanisms. Further investigation on the production of cytokinin by our *Bacillus* spp. will provide a better understanding of the relationship between PGPR and plant root development.

In conclusion, the Petri dish system is a potential method to study the interaction between PGPR and plant root architecture. *Bacillus* spp. reduced Arabidopsis total root length but enhanced root hair length in a dose-dependent manner. The mechanism of bacilli elicitation of growth promotion may involve the enhancement of root hair development and therefore increase nutrient and water uptake.

Table 1. Effect of PGPR on total root length of 4-day-old Arabidopsis.

Treatment	Total root length (cm) PGPR concentration (cfu/ml)[a]			
	10^6	10^7	10^8	10^9
Control	7.72^{ab}	7.72^{bc}	7.72^{a}	7.72^{a}
89B-61	8.41^{a}	8.80^{ab}	4.03^{b}	3.07^{b}
90-166	6.22^{bc}	10.02^{a}	6.80^{a}	1.72^{b}
C9	6.07^{bc}	4.32^{de}	2.92^{bc}	-
GB03	5.61^{cd}	4.43^{d}	1.82^{cd}	-
IN937a	3.96^{de}	2.48^{e}	-	-
INR7	3.09^{e}	3.93^{de}	1.60^{d}	-
SE34	7.08^{abc}	6.36^{c}	1.60^{d}	-
T4	6.72^{abc}	3.57^{de}	1.85^{cd}	-
LSD ($P = 0.05$)	1.76	1.88	1.14	1.62

[a] Different concentrations of PGPR (10^6 to 10^9 cfu/ml) were mixed with plant medium before seeding of Arabidopsis. Values are means of 20 replications. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$.

- Indicates no measurable root length.

Table 2. Effect of PGPR on total root length of 7-day-old Arabidopsis.

Treatment	Total root length (cm) PGPR concentration (cfu/ml)[a]			
	10^6	10^7	10^8	10^9
Control	32.17^{ab}	32.17^{b}	32.17^{a}	32.17^{a}
89B-61	33.91^{a}	42.91^{a}	18.89^{b}	8.71^{b}
90-166	15.71^{cde}	28.97^{b}	18.25^{b}	2.43^{b}
C9	12.89^{ef}	11.38^{cd}	4.57^{c}	-
GB03	13.15^{dfe}	7.73^{cd}	2.06^{c}	-
IN937a	5.77^{f}	5.66^{d}	2.52^{c}	-
INR7	11.08^{ef}	7.78^{cd}	-	-
SE34	22.11^{cd}	16.29^{c}	5.74^{c}	-
T4	23.65^{bc}	7.96^{cd}	3.96^{c}	-
LSD (P = 0.05)	9.11	8.57	7.24	11.62

[a] Different concentrations of PGPR (10^6 to 10^9 cfu/ml) were mixed with plant medium before seeding of Arabidopsis. Values are means of 20 replications. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at P = 0.05.

- Indicates no measurable root length.

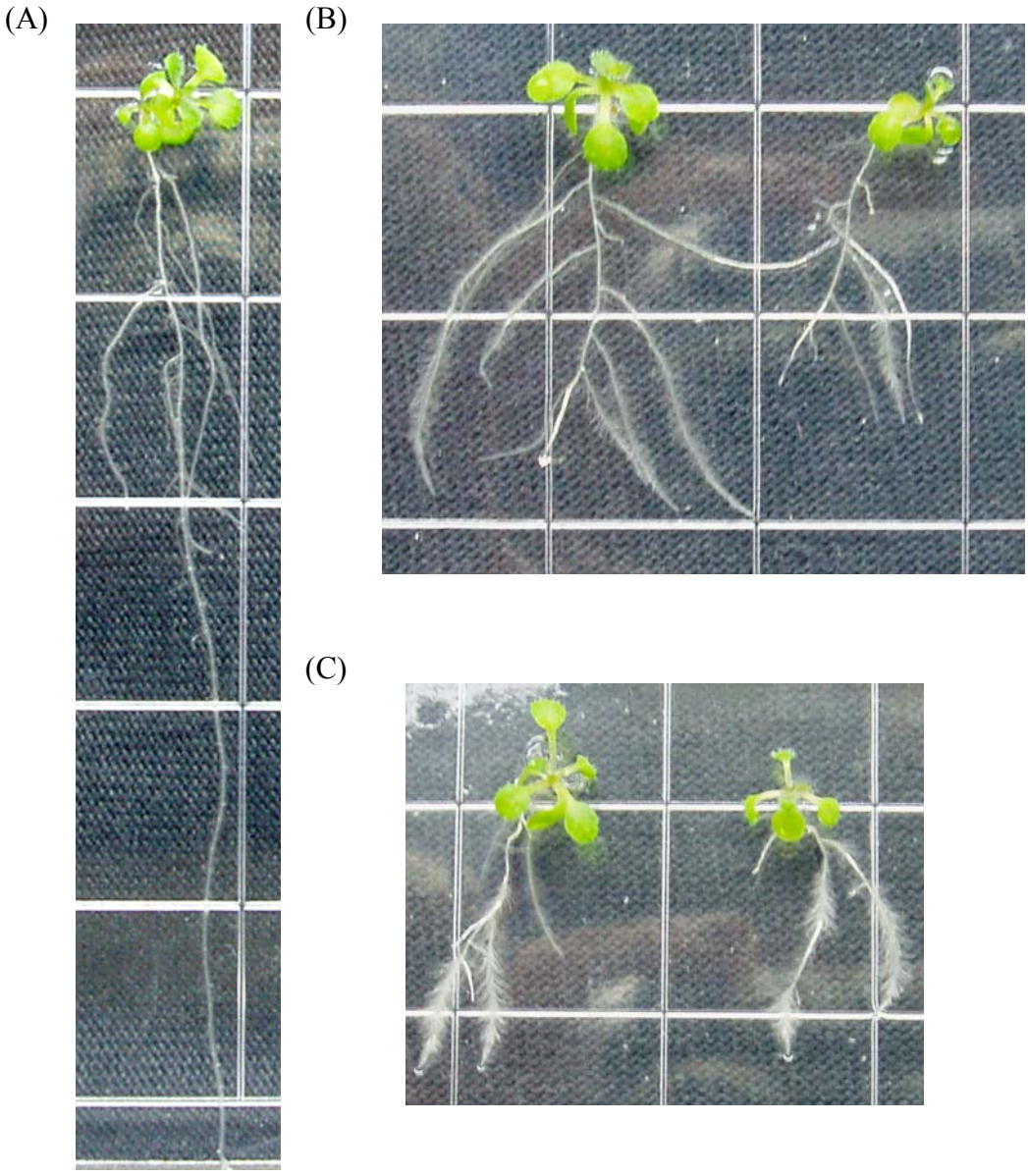


Figure 1. Root morphology of 14-day-old Arabidopsis. (A) Water control. (B) *Bacillus* strain C9 at 10^6 cfu/ml. (C) *Bacillus* strain C9 at 10^7 cfu/ml.

(A)

(B)

(C)

Figure 2. Stimulation of root hairs by bacilli on 14-day-old Arabidopsis. (A) Water control. (B) *Bacillus* strain INR7 at 10^6 cfu/ml. (C) *Bacillus* strain INR7 at 10^7 cfu/ml.

IV. SALT STRESS TOLERANCE BY PGPR

INTRODUCTION

Environmental stresses are major limiting factors for agricultural productivity worldwide (Cherry, 1987). These stresses decrease yields of crops and also represent barriers to the introduction of crop plants into areas that are not suitable for crop cultivation. Abiotic stress factors include high and low temperature (Iba, 2002; Sung et al., 2003), salinity (Hasegawa et al., 2000; Mansour and Salama, 2004), drought (Ingram and Bartels, 1996; Munns, 2002; Zhu, 2002), flooding (Dat et al., 2004; Drew, 1997), ultraviolet light (Gyula et al., 2003; Stratmann, 2003), air pollution (ozone) (Langebartels et al., 2002), and heavy metals (Jackson et al., 1990; Schutzendubel and Polle, 2002). The yield losses associated with abiotic stresses can reach 50% to 82%, depending on the crop (Bray et al., 2000).

In many semi-arid and arid regions of the world, crop yield is limited due to increasing salinity of irrigation water as well as soil salinity. Under high salinity, plants exhibit decreased water uptake, and they subsequently exhibit a reduced leaf growth rate, which restricts photosynthetic capacity (Munns, 2002). Plant responses to salt stress and water deficiency (drought) have much in common and involve a number of metabolic and physiological changes, many of which have not been fully characterized. Abscisic acid (ABA) is the major plant hormone involved in the response to salinity, drought, and cold

stress (Himmelbach et al., 2003; Leung and Giraudat, 1998). Under drought and salt stress, endogenous levels of ABA increase dramatically in all plant tissues. The role of ABA in drought and salt stress is to increase cellular dehydration tolerance and maintain water balance (Zhu, 2002).

Several secondary metabolites of plants have been implicated in signaling in responses to a variety of abiotic and biotic stresses. Salicylic acid (SA) is involved in response to systemic acquired resistance (SAR) and the hypersensitive response (HR) (Durrant and Dong, 2004; Klessig and Malamy, 1994; Lamb and Dixon, 1997; Shah, 2003). Besides its role in biotic stresses, SA may modulate plant responses to several abiotic stresses. Salicylic acid levels increase during exposure to ozone or ultraviolet light (Sharma et al., 1996; Yalpani et al., 1994), while pretreatment with SA can protect plants from paraquat-induced oxidative stress (Strobel and Kuc, 1995). Dat et al. (1998) demonstrated the induction of thermotolerance by spraying SA on mustard seedlings. Similarly, wheat (Shakirova et al., 2003) and tomato (Tari et al., 2002) pre-treated with SA exhibit tolerance to salinity stress. In maize, SA reduces the effects of chilling injury (Janda et al., 1999) but increases sensitivity to drought (Nemeth et al., 2002).

There are overlaps in signal transduction pathways between abiotic and biotic stresses. Studies have shown that plants resistant to one stress are often more resistant to others. This phenomenon is known as cross-tolerance (Bowler and Fluhr, 2000; Pastori and Foyer, 2002). For example, ozone treatment triggers induced resistance in Arabidopsis to subsequent infection with *Pseudomonas syringae* (Sharma et al., 1996). In tomato, salt stress induces accumulation of proteinase inhibitors and activates expression of other wound-related genes. Salt stress strongly enhances the wounding response both

locally and systemically (Dombrowski, 2003). Moreover, cool-season grasses infected with endophytic fungi have enhanced tolerance to drought and mineral stresses (Malinowski and Belesky, 2000).

Application of plant growth-promoting rhizobacteria (PGPR) increases plant health overall. Precisely how the interaction of plants and PGPR affect plant physiology and metabolism is unclear. There is much research concerning the effect of PGPR on a plant's resistance or tolerance to different plant pathogens (Bakker et al., 2003; Bloemberg and Lugtenberg, 2001; van Loon et al., 1998). In contrast, only a few PGPR strains have been studied for their capacity to enhance plant tolerance of environmental stresses. Plants with reduced levels of ethylene, resulting from inoculation of PGPR that produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, showed tolerance to flooding stress (Grichko and Glick, 2001b) and metal contaminants (Belimov et al., 2005; Burd et al., 1998; Nie et al., 2002). PGPR strain *Paenibacillus polymyxa* B2 protected *Arabidopsis* against *Erwinia carotovora* (biotic stress) and drought (abiotic stress) (Timmusk and Wagner, 1999). In field experiments, sorghum plants inoculated with *Azospirillum brasilense* Cd had a 15-18% grain yield increase compared to noninoculated plants under dryland conditions (Sarig et al., 1988). *Azospirillum*-inoculated sorghum plants had more water content, higher water potential, and lower canopy temperature in their foliage. Hence, they were less drought-stressed than noninoculated plants.

In nature plants are often exposed to multiple stresses, and their response to a variety of stresses determines their capacity to survive. The development of PGPR that enhance stress tolerance in plants may be a promising new strategy for sustainable agricultural productions. The objective of this study was to test the hypothesis that some

PGPR strains that promote vegetable growth and yield and induce systemic disease resistance in field trials can activate salt stress tolerance.

MATERIALS AND METHODS

PGPR strains and inoculum preparation.

Eight different PGPR strains were used in this study: *Pseudomonas fluorescens* 89B-61, *Serratia marcescens* 90-166, *Bacillus pasteurii* C9, *B. subtilis* GB03, *B. amyloliquefaciens* IN937a, *B. pumilus* INR7, *B. pumilus* SE34, and *B. pumilus* T4. All PGPR strains were maintained in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) amended with 20% glycerol at –80°C. PGPR inoculum was prepared by harvesting bacterial cells from 24h cultures on tryptic soy agar (TSA) plates at 28°C. The inoculum was suspended in sterile distilled water to yield 10^9 colony forming units (cfu) per ml. One ml of PGPR suspension was applied to each tomato seed.

Plant material and growth conditions.

Tomato plants (*Lycopersicon esculentum* cv. Sanibel) were grown in Pro-Mix soilless media (Premier Horticulture, Riviere-du-Loup, Quebec, Canada) in greenhouse conditions with natural light at 22 to 25 °C and 70 to 80% relative humidity. Tomato plants were sown into 10 x 10 cm pots with 2 plants per pot.

Salt treatments.

Three different salt concentrations (0, 100, and 200 mM) were applied at three dates: day-0, day-7, and day-14. A salt solution of 50 ml was applied to each pot three

days in a row without watering. The experiment was designed as a randomized complete block with 9 (8 PGPR strains and a water control) PGPR treatments × 3 salt treatments × 3 salt application times. Ten replications per treatment were used. The experiment was conducted three times.

The percentage of plants emerged was determined in each treatment at 12 and 21 days after seeding. Shoot fresh and dry weight, root fresh and dry weight, and SPAD chlorophyll meter reading were measured after 35 days. SPAD reading was measured by a hand-held chlorophyll meter (SPAD 502, Minolta Co. Ltd., Japan) on two fully expanded leaves (the 3rd and 4th leaflets from the top) per plant. Several reports have shown the linear relationship between SPAD readings and leaf chlorophyll content (Cartelat et al., 2005; Kantety et al., 1996; Wu et al., 1998).

PGPR population affected by salt stress.

An initial bacterial population of 10^2 cfu was added into 50 ml tryptic soy broth (TSB) containing sterilized water, 100 mM NaCl, or 200 mM NaCl solution for 24 h incubation at room temperature at 200 rpm. The bacterial population was calculated using the most probable number (MPN) procedure (Alexander, 1982). A standard ten-fold serial dilution was conducted, and the numbers of positive growth responses were recorded after incubation for 3 days. Three replications per treatment were used.

Statistical analysis.

All data were subjected to analysis of variance (ANOVA) using JMP software (SAS Institute, Cary, NC). Treatment means were separated by Fisher's protected least significant difference (LSD) test at $P = 0.05$.

RESULTS

PGPR effects on percentage of emergence of tomato under salt stress.

Tomato plants without PGPR treatment were subject to salt stress (100 mM or 200 mM NaCl) at 0, 7, and 14 days after seeding (DAS), and the percentage of emerged plants was recorded at 12 DAS (Figure 1). Emergence was most affected when seeds were subject to 200 mM NaCl at 0 DAS. When 100 mM NaCl was applied at 0 and 7 DAS, the emergence was significantly lower than the water control. However, there was no significant effect on emergence with 200 mM NaCl at 7 DAS. When PGPR-treated tomato seeds were subject to high salt stress (200 mM) at 0 DAS, two *Bacillus* strains C9 and INR7, significantly enhanced emergence at 21 DAS (Figure 2).

PGPR effects on tomato shoot growth under salt stress.

Without PGPR treatment, tomato plants had a 73.1 % and 34.1 % reduction of shoot weight when 200 mM NaCl was applied at 0 and 7 DAS, respectively, and the shoot weight was significantly decreased compared to the no-salt control ($LSD_{0.05} = 0.69$) (Table 1). *Bacillus* strains INR7 and SE34 significantly enhanced shoot weight when 100 mM NaCl was applied at 14 DAS and 200 mM NaCl was applied at 0 DAS. Another *Bacillus* strain, T4, significantly increased shoot weight when 100 mM NaCl was applied at 7 and 14 DAS and 200 mM NaCl was applied at 7 DAS. Moreover, when 200 mM

NaCl was applied at 0 DAS, INR7- and SE34-treated tomato plants had 55.1% and 59.6% reduction of shoot weight, respectively. With T4-treated tomato plants, the application of 200 mM NaCl at 7 DAS resulted in only a 17.3 % reduction of shoot weight. However, when 200 mM NaCl was applied at 14 DAS, 4 PGPR strains, 89B-61, 90-166, C9, and IN937a, significantly reduced shoot weight compared to the water control (Table 1).

PGPR effects on tomato root growth under salt stress.

When 200 mM NaCl was applied at 0 DAS, tomato plants treated with *Bacillus* strains INR7 and T4 had significantly greater root weights compared to the nonbacterized salt control (Table 2). When 200 mM NaCl was applied at 7 DAS, *Bacillus* strain T4 still significantly increased root weight. With 200 mM NaCl applied at 0 DAS, nonbacterized tomato plants had 92.4% reduction on root weight compared to no-salt control. Under the same condition, INR7-treated plants had 82.4% reduction on root weight compared to no-salt INR7-treated plants. T4-treated plants had 76.6% reduction and only 50% reduction on root weight compared to no-salt T4-treated plants when 200 mM NaCl were applied at 0 and 7 DAS, respectively. However, when 200 mM NaCl was applied at 14 DAS, all 8 PGPR strains significantly reduced root weight compared to the nonbacterized salt control (Table 2).

Five *Bacillus* strains, C9, GB03, INR7, SE34, and T4, were chosen for further study on the basis of consistent performance (Table 3). *Bacillus* strain C9 and T4 significantly enhanced root fresh and dry weight when 100 mM NaCl was applied at 7 DAS. However, when 200 mM NaCl was applied at 0 DAS, C9 significantly reduced

root growth. *Bacillus* strain SE34 significantly enhanced root dry weight when 100 mM NaCl was applied at 0 and 7 DAS.

PGPR effects on tomato SPAD chlorophyll meter reading under salt stress.

Four *Bacillus* strains, GB03, INR7, SE34 and T4, significantly enhanced tomato SPAD chlorophyll meter reading without the presence of salt (Table 4). When 100 mM NaCl was applied at 0 DAS, *Bacillus* strains C9, INR7, SE34, and T4 significantly increased SPAD reading compared to the nonbacterized salt control. There was no significant effect on the chlorophyll SPAD reading under other salt stress conditions.

Effect of salt on population densities of PGPR.

Additive salt in TSB medium did not affect the growth of PGPR (Table 5). Moreover, *Bacillus* strains INR7 and SE34 significantly increased their population size under both 100 and 200 mM NaCl TSB medium.

DISCUSSION

Our results indicate that some PGPR strains can ameliorate salt stress. High salt stress (200 mM) significantly reduced the emergence of tomato plants when salt was applied at the time of seeding (0 DAS) (Figure 1). However, the percentage of emergence was significantly increased upon treatment with the *Bacillus* strains C9 and INR7 (Figure 2), while emergence was not affected with other strains. Hence, promotion of seedling emergence in the presence of salt is strain-specific. Interestingly, in the absence of salt

stress, PGPR did not have a significant effect on the percentage emergence (data not shown).

After seedling emergence, three *Bacillus* strains, INR7, SE34, and T4, promoted shoot growth under salt stress (Table 1). Our results showed that *Bacillus* spp. PGPR provided a better growth promotion compared to Gram-negative PGPR when tomato plants were under salt stress. Fischer et al. (2000) had shown that salt treatment impaired Gram-negative PGPR, *Azospirillum brasilense* Cd, colonization of wheat. This result suggested that salt treatment may alter the colonization between PGPR strains and plant root and cause a deleterious effect on plant growth. Therefore, the capacity of PGPR colonization on roots may be an important characteristic for screening PGPR that can enhance plant salt tolerance.

Under salt stress, PGPR seems to have little or no effect on tomato root growth. In our experiments, only *Bacillus* strain T4 showed a consistent growth promotion effect on root growth under salt stress (Table 2 and 3). Further research on its colonization on tomato roots may provide more understanding on salt tolerance enhancement by PGPR. However, when 200 mM NaCl was applied at 14 DAS, PGPR-treated tomato plants had significantly lower root weight compared to nonbacterized salt control (Table 2). This deleterious effect may be caused by osmotic potential changes that interfered with the mineral and water uptake. Somehow bacteria-treated tomato plant was more susceptible to salt after seedling emerged. The colonization of PGPR may be most affected at this stage.

Regarding the effect on PGPR population by salt, our results showed that salt treatment did not reduce the population size of PGPR in culture condition (Table 5).

However, two *Bacillus* strains, INR7 and SE34, had significantly higher population in 100 and 200 mM NaCl containing medium. Further research on the PGPR population density in rhizosphere will enhance our knowledge on the aspect of salt stress tolerance.

We used SPAD readings to understand further how PGPR affect plants under salt stress. A positive correlation ($r^2 > 0.95$) between SPAD chlorophyll meter readings, extractable chlorophyll, and tissue nitrogen concentrations had been reported (Kantety et al., 1996). Previous work indicated that PGPR can increase the chlorophyll content of plants, which is not surprising, considering that some PGPR enhanced plant nitrogen uptake (Dobbelaere et al., 2003; Lucy et al., 2004). Decreases in chlorophyll content of plants growing under salt stress has also been reported (Munns, 2002). Hamdia and El-Komy (1997) demonstrated that *Azospirillum* could ameliorate this salt-related reduction in chlorophyll content on maize. Our results differ from these past reports and support the conclusion that PGPR can improve chlorophyll content under salt stress. While nonbacterized tomato did not exhibit lower SPAD readings under salt stress (Table 4), treatment with four PGPR strains with 100 mM NaCl significantly enhanced SPAD readings compared to the nonbacterized control under the same salt level. The increase in chlorophyll content that we observed in nonbacterized tomato under salt stress supports the findings by Murillo-Amador et al. (2002) on cowpea.

In this study, *Bacillus* spp. ameliorated tomato emergence, shoot growth and chlorophyll content under lower level of salt stress. Application of PGPR to enhance stress tolerance in plants is a feasible strategy for improving crop productions in salinity environment.

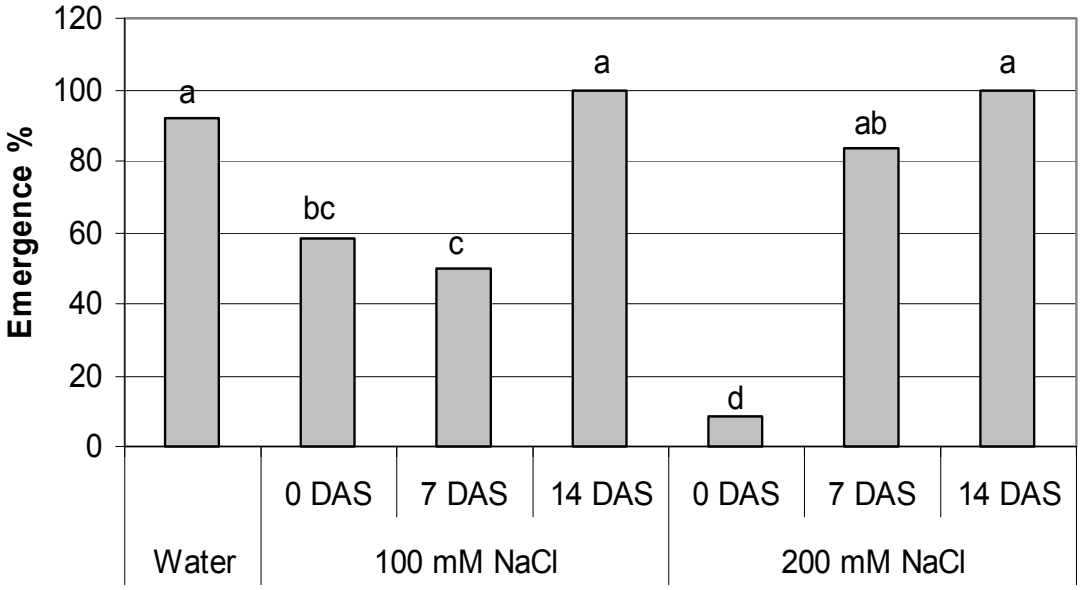


Figure 1. Effect of salt stress (100 mM or 200 mM NaCl) applied at 0, 7, and 14 days after seeding (DAS) on percentage of emergence of tomato plants at 12 DAS. Values are the means of 10 replications (LSD = 31.06). Different letters indicate significant differences using Fisher's LSD test at $P = 0.05$.

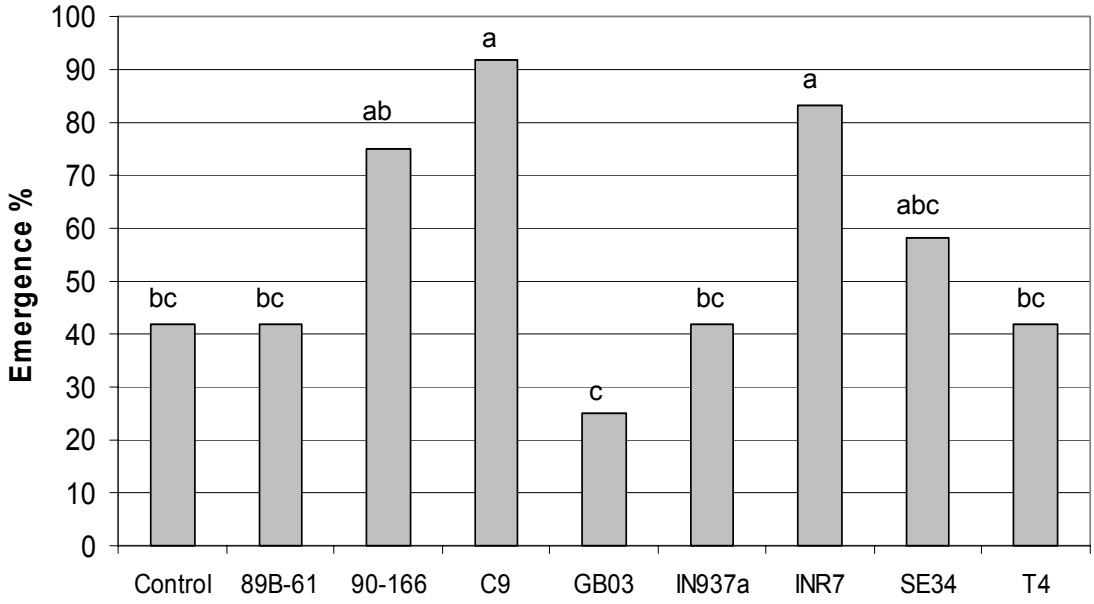


Figure 2. Effect of 200 mM NaCl salt stress applied at 0 days after seeding (DAS) on percentage of emergence of tomato plants at 21 days after seeding. Values are the means of 10 replications (LSD = 40.03). Different letters indicate significant differences using Fisher's LSD test at $P = 0.05$.

Table 1. Effect of salt stress on PGPR-treated tomato shoot weight (g) at 35 days after seeding.

Treatment	Water	100 mM NaCl			200 mM NaCl		
		0 DAS	7 DAS	14 DAS	0 DAS	7 DAS	14 DAS
Control	3.31^{b}	3.11^{a}	2.75^{bc}	3.23^{bc}	0.89^{bc}	2.18^{bc}	2.96^{a}
89B-61	3.66^{ab}	2.76^{ab}	2.39^{c}	3.04^{bc}	0.91^{bc}	1.60^{c}	2.35^{bc}
90-166	**4.02^{a}**	2.17^{b}	3.24^{abc}	3.37^{abc}	1.36^{ab}	2.34^{bc}	2.22^{c}
C9	3.32^{b}	2.38^{ab}	3.00^{abc}	3.54^{ab}	1.32^{ab}	1.77^{bc}	2.49^{bc}
GB03	3.86^{ab}	3.04^{a}	3.26^{abc}	3.52^{ab}	0.47^{c}	1.84^{bc}	2.67^{ab}
IN937a	3.62^{ab}	2.57^{ab}	2.58^{c}	3.80^{c}	0.72^{c}	2.06^{bc}	2.43^{bc}
INR7	3.83^{ab}	2.68^{ab}	3.18^{abc}	**3.93^{a}**	**1.72^{a}**	2.59^{ab}	3.02^{a}
SE34	3.84^{ab}	2.78^{ab}	3.57^{ab}	**4.01^{a}**	**1.55^{a}**	2.25^{bc}	3.01^{a}
T4	**3.92^{a}**	3.19^{a}	**3.84^{a}**	**3.91^{a}**	1.48^{ab}	**3.24^{a}**	2.97^{a}
LSD (P = 0.05)	0.57	0.82	0.92	0.64	0.59	0.82	0.44

Values are means of 10 replications. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$. Bold letters indicate values that are significantly greater than control.

Table 2. Effect of salt stress on PGPR-treated tomato root weight (g) at 35 days after seeding.

Treatment	Water	100 mM NaCl			200 mM NaCl		
		0 DAS	7 DAS	14 DAS	0 DAS	7 DAS	14 DAS
Control	1.19^{a}	0.50^{abc}	0.71^{bc}	0.90^{ab}	0.09^{cd}	0.39^{b}	0.76^{a}
89B-61	1.27^{a}	0.49^{abc}	0.41^{d}	0.93^{a}	0.11^{bcd}	0.27^{b}	0.51^{bcd}
90-166	1.16^{a}	0.22^{d}	0.68^{bcd}	0.70^{bcd}	0.17^{bc}	0.33^{b}	0.36^{de}
C9	0.93^{b}	0.37^{cd}	0.52^{bcd}	0.58^{d}	0.15^{bcd}	0.27^{b}	0.34^{e}
GB03	1.07^{ab}	0.58^{ab}	0.55^{bcd}	0.86^{abc}	0.04^{d}	0.26^{b}	0.49^{bcde}
IN937a	1.17^{a}	0.44^{bc}	0.46^{cd}	0.68^{cd}	0.08^{cd}	0.32^{b}	0.41^{cde}
INR7	1.19^{a}	0.43^{bc}	0.55^{bcd}	0.94^{a}	**0.21^{ab}**	0.41^{b}	0.54^{bc}
SE34	1.13^{ab}	0.54^{abc}	0.77^{b}	0.78^{abcd}	0.18^{abc}	0.38^{b}	0.60^{b}
T4	1.24^{a}	0.64^{a}	**1.06^{a}**	0.78^{abcd}	**0.29^{a}**	**0.62^{a}**	0.59^{b}
LSD (P = 0.05)	0.22	0.19	0.28	0.21	0.11	0.20	0.15

Values are means of 10 replications. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$. Bold letters indicate values that are significantly greater than control.

Table 3. Effect of salt stress on PGPR-treated tomato root fresh and dry weight (mg) at 35 days after seeding.

Treatment	Water		100 mM NaCl				200 mM NaCl			
			0 DAS		7 DAS		0 DAS		7 DAS	
	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight
Control	865.0^{bc}	57.3^{b}	838.3	52.5^{b}	568.3^{bc}	32.2^{c}	783.3^{a}	52.7	480.0	23.5
C9	845.0^{bc}	59.5^{b}	1101.7	76.0^{ab}	**1161.7^{a}**	**71.0^{ab}**	210.0^{b}	12.3	578.3	23.4
GB03	**1255.0^{a}**	**85.7^{a}**	1186.7	70.7^{ab}	921.7^{ab}	55.5^{abc}	743.3^{a}	45.7	401.7	26.3
INR7	1096.7^{abc}	**81.8^{a}**	1295.0	85.3^{ab}	378.3^{c}	37.0^{bc}	718.3^{ab}	48.0	678.3	40.0
SE34	1120.0^{ab}	**83.0^{a}**	1203.3	**93.8^{a}**	896.7^{ab}	**84.5^{a}**	878.3^{a}	53.2	440.0	49.5
T4	830.0^{c}	59.2^{b}	940.0	64.0^{ab}	**1246.7^{a}**	**78.3^{a}**	615.0^{ab}	42.3	458.3	41.3
LSD (P = 0.05)	289.0	21.2	486.1	36.1	510.3	36.1	520.0	36.9	656.8	44.4

Values are means of 10 replications. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$. Bold letters indicate values that are significantly greater than control.

Table 4. Effect of salt stress on SPAD chlorophyll meter reading of PGPR-treated tomato plants at 35 days after seeding.

Treatment	Water	100 mM NaCl		200 mM NaCl	
		0 DAS	7 DAS	0 DAS	7 DAS
Control	20.46[c]	23.19[b]	30.83	29.73	30.51
C9	18.28[c]	**30.16[a]**	29.52	28.74	29.85
GB03	**25.24[b]**	21.88[b]	28.85	29.88	29.16
INR7	**29.30[a]**	**31.50[a]**	30.59	29.68	30.09
SE34	**27.79[ab]**	**31.01[a]**	31.08	28.87	29.13
T4	**28.98[ab]**	**31.62[a]**	29.92	29.14	29.84
LSD (P = 0.05)	3.93	2.87	3.11	2.55	2.78

Values are means of 10 replications. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$. Bold letters indicate values that are significantly greater than control.

Table 5. Effect of salt on population densities of PGPR.

Treatment	PGPR population (log(cfu/ml))			LSD ($P = 0.05$)
	0 mM NaCl	100 mM NaCl	200 mM NaCl	
INR7	8.57	**>9.04**	**8.92**	0.31
SE34	7.24	**7.92**	**8.57**	0.42
T4	7.54	7.98	7.67	0.87

Values are means of 3 replications. Bold letters indicate values that are significantly greater than control (0 mM NaCl) using Fisher's LSD test at $P = 0.05$.

V. EFFECT OF COMMERCIAL PGPR PRODUCTS ON GROWTH, YIELD, AND FRUIT QUALITY OF TOMATO UNDER FIELD CONDITIONS

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are free-living rhizobacteria that colonize plant roots and are beneficial (mutualistic) to plants (Glick et al., 1999; Kloepper, 1994). Plant growth benefits resulting from PGPR application include increases in germination rate, root and shoot weight, lateral root growth, leaf surface area, chlorophyll content, nitrogen content, and yield. In general, yield is enhanced up to 10% for cereal crops and 15 to 50% for different vegetable crops with PGPR applications (Kloepper, 1994). A growing number of PGPR are being commercialized as biocontrol agents or biofertilizers in the U.S. (Glick et al., 1999; Kloepper et al., 2004b; McSpadden Gardener and Fravel, 2002). In recent years *Bacillus* spp. have drawn more attention for commercial biofertilizers due to their production of heat- and desiccation-tolerant endospores under stress environments (Emmert and Handelsman, 1999; Handelsman and Stabb, 1996). One of the major challenges for large-scale applications of PGPR products is to maintain high populations of living microorganisms for longer shelf-life. Several commercially available biofertilizer products, such as AgBlend®, BioYield®, and Equity™, include *Bacillus* spp. to facilitate the formulation process.

The majority of tomato production in Alabama is performed using transplants of 4 to 5-week-old plants. Such transplants are susceptible to plant pathogens and environmental stresses, especially in the first two weeks after transplanting. Transplant shock is a period in which newly unconditioned planted transplants adjust to their new environment. During this shock period, roots of plants begin growing in the field while no new leaf growth occurs. Kloepper et al. (2004a) has shown that PGPR elicited growth promotion, reduced transplant shock, and stimulated rooting with several vegetable transplant system. Another field evaluation experiment showed that PGPR enhanced transplant growth, vigor, and survival in both tomato and pepper transplants (Kokalis-Burelle et al., 2002). Hence, transplants with PGPR treatment exhibited faster growth of new leaves.

Many tomato growers are choosing organic methods because of a higher premium in the marketplace (http://attra.ncat.org/attra-pub/tomato.html). The amount of nitrogen application for vigorous growth of tomato is critical. Fresh market tomatoes require 75 to 100 pounds of nitrogen per acre. Organic fertilizers and composts are typically much lower in nitrogen content and hence, additional supplemental nitrogen is needed for organic tomato production. Even when the level of organic fertilizer applied is calculated to deliver the same nitrogen level as inorganic fertilizers, plants supplied with organic fertilizer often grow under conditions of nitrogen stress because the nitrogen in organic fertilizers is released more slowly than from inorganic fertilizers.

We previously demonstrated that PGPR can ameliorate various environmental stresses. For example, *Bacillus* spp. ameliorated tomato emergence, shoot growth and chlorophyll content under lower level of salt stress. Moreover, under high temperature

Bacillus spp. increased the tomato seedling survival rate and enhanced the shoot weight (Hu and Kloepper, 2004). In this study, we were interested whether transplant stress and nitrogen stress resulting from the use of organic fertilizer could be overcome via use of commercially available PGPR.

MATERIALS AND METHODS

Field trials.

Two field trials were conducted in summer 2004: one at the Sand Mountain Research and Extension Center in Crossville, AL and one at the North Alabama Horticulture Research Center in Cullman, AL. Commercially prepared tomato transplants (*Lycopersicon esculentum* cv. Florida 47) were used in both field trials. The experiment was 2 × 4 factorial (2 factors × 4 treatments). Factors were inorganic and organic fertilizers. Treatments were AgBlend®, Equity™, BioYield® and a noninoculated control. AgBlend®, obtained from Advanced Microbial Systems in Pilot Point, TX, is a liquid product produced by continuous anaerobic fermentation that contains multi-trophic communities of culturable and nonculturable bacteria, algae, cyanobacteria, and protozoa. Equity™, obtained from Naturize Inc. in Jacksonville, FL, is a liquid formulation of 47 strains of bacilli. Both liquid products were applied at the label rate of 2 quarts per acre (47.5 L/ha) at the time of transplanting. BioYield® is a spore preparation of PGPR strains GB03 (*B. subtilis*) and IN937a (*B. amyloliquefaciens*) on chitosan flakes. To treat plants with BioYield, the product was mixed 1:40 (v : v) into the soilless potting mix used to prepare the transplants in the greenhouse. Organic fertilizer, provided by Daniels® Plant

Food, Inc. in Sherman, TX, is a liquid formulation of 10-4-3 NPK. Treatments were arranged as a RCB within factors with six replications and ten plants per replication.

At 2, 4, and 7 weeks after transplanting, the mean growth index was calculated for each treatment by multiplying the height × width of four plants per replication. Cumulative yield was determined by multiple hand pickings of fruit at maturity. The weight of marketable fruit was recorded by picking from all 10 plants per replication.

For tomato fruit quality analysis, ten tomato fruits were collected from each replication. A total of 60 fruits per treatment were selected from the field located at the Sand Mountain center.

Initial pH and titratable acidity.

Initial pH and titratable acidity were measured using an automated titrimeter (Metrohm Titrino Model 751 GPD and Metrohm Sample Changer, Metrohm Corp., Herisau, Switzerland) and computer software (Brinkmann Titrino Worcell 4.4 Software, Brinkmann Corp., Westbury, NY). Ten grams of frozen tomato sample were added into a pre-chilled Virtis shear beaker containing 40 ml of deionized distilled water and homogenized for 1 min with a Virtis shear homogenizer (Virtishear, model 225318, Gardiner, NY). The homogenate was stirred at 300 rpm for 10 min and centrifuged at 15,000 g for 20 min. All procedures were conducted at 4 °C. After being centrifuged, the supernatant was filtered with Miracloth (Calbiochem, La Jolla, Ca), and the final volume was brought to 100 ml with deionized distilled water. Ten ml of diluted supernatant were then placed into auto titrator sample cups to measure initial pH and acidity. Acidity was titrated as the percent citric acid equivalent using 0.1 N sodium hydroxide.

Total soluble solid (TSS) and titratable acidity/TSS ratio.

Total soluble solids (TSS) were determined using a hand-held refractometer (Leica Refractometer, model 10494, Buffalo, NY). One drop (approximately 500 µl) of homogenate from the previous procedure was placed on the refractometer. The TSS/TA ratio was calculated by dividing total soluble solid values by the titratable acidity values of each sample.

Vitamin C analysis.

The procedure for ascorbic acid (vitamin C) analysis was modified from Gossett et al. (1994). Ten grams of frozen pericarp tomato sample were homogenized in 40 ml of pre-chilled extraction solution containing 3% phosphoric acid and 8% acetic acid for 1 min. Samples were kept on ice in diffused light to reduce auto oxidation of vitamin C. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C, and the supernatant was filtered with Miracloth. A 5 ml aliquot of supernatant was passed through a previously conditioned SEP-PAK cartridge (Waters Associates, Milford, MA), and the last 3 ml of sample was collected for further analysis. The SEP-PAK cartridge was conditioned with acetonitrile and washed with deionized distilled water.

To determine the reduced ascorbate (AA), 200 µl of SPE extract was mixed with 500 µl of 150 mM KH_2PO_4 buffer containing 5 mM EDTA (pH 7.4) and 100 µl deionized distilled water. After 10 min incubation at root temperature, 400 µl of 10% trichloroacetic acid (TCA), 400 µl of 44% o-phosphoric acid, 400 µl of 4% dipyridyl, and 200 µl $FeCl_3$ were added into reaction mixture and incubated for 2 hours at 40°C.

To determine total ascorbic acid (reduced ascorbate (AA) and oxidized ascorbate (DHA)), 200 µl of extract was mixed with 500 µl of 150 mM KH_2PO_4 buffer containing 5 mM EDTA (pH 7.4) and 100 µl 10 mM dithiothreitol (DTT). After 10 min incubation at room temperature, 100 µl of 0.5% N-ethylmaleimide, 400 µl of 10% TCA, 400 µl of 44% o-phosphoric acid, 400 µl of 4% dipyridyl, and 200 µl 3% $FeCl_3$ were added into reaction mixture and incubated for 2 hours at 40°C. The absorbance was measured at 525 nm using BIO-TEK microplate reader (SYNERGY HTTR-I, model 185956, Winooski, VT).

Statistical analysis.

All data were subjected to analysis of variance (ANOVA) using JMP software (SAS Institute, Cary, NC). Treatment means were separated by Fisher's protected least significant difference (LSD) test at $P = 0.05$.

RESULTS

PGPR effect on tomato growth in combination with organic and inorganic fertilizer.

At all sample times except 2 weeks after transplanting (WAT) the plant growth index of the inorganic nonbacterized control was significantly higher than that of the organic nonbacterized control (Tables 1 and 2). This supports the premise that plants that received the organic fertilizer were stressed relative to those that received inorganic fertilizer.

PGPR treatments consistently resulted in significantly higher plant growth indices compared to both the organic and inorganic nonbacterized controls (Tables 1 and 2). Moreover, PGPR with inorganic fertilizer also had significantly higher plant growth

index compared to PGPR with organic fertilizer, especially in the field trial in Cullman (Table 2).

Both the organic and inorganic nonbacterized controls exhibited transplant shock in the first two weeks after transplanting. PGPR treatments reduced the effect of transplant shock by increasing growth of transplants during the first two weeks (Figure 1, Tables 1 and 2).

PGPR effects on marketable tomato yield.

At the Sand Mountain center, there were no significant differences among treatments on the yield of tomato regarding total marketable weight and number of marketable fruit (Table 3). However, the inorganic nonbacterized control had significantly higher weight per fruit than the organic nonbacterized control. Two-way factorial analysis showed no PGPR treatment effect and no interaction between factors and treatments. Hence, all treatments were combined by the factors. Inorganic fertilized tomato plants had significantly greater weight per fruit than did organic fertilized plants ($P = 0.0043$).

At the Cullman center, the inorganic nonbacterized control had significantly higher total marketable weight and weight per fruit compared to the organic nonbacterized control (Table 4). Two-way factorial analysis also showed no PGPR treatment effect and no interaction between factors and treatments. Hence, all treatments were combined by the factors. Inorganic fertilized tomato plants had significantly higher total market weight ($P = 0.0002$), higher total market number ($P = 0.0046$), and higher weight per fruit ($P < 0.0001$) than organic fertilized plants.

PGPR effect on tomato fruit flavor.

A key aspect of tomato quality is flavor, which is mainly determined by the sugar (total soluble solids, TSS) and acidity composition of the fruit. Tomatoes treated with AgBlend and organic fertilizer had significantly higher TSS than the inorganic nonbacterized control (Table 5). Two-way factorial analysis showed no PGPR treatment effect and no interaction between factors and treatments on TSS. Hence, all treatments were combined by the factors. Organic fertilized tomato plants had significantly higher TSS than inorganic fertilized plants ($P = 0.0247$).

Initial pH ranged from 5.67 to 5.76 with no significant difference among all treatments. There was no significant difference of titratable acidity (TA) among the treatments.

Higher TSS/TA ratio means sweeter flavor. With the organic fertilizer, AgBlend- and BioYield-treated tomatoes had significantly higher TSS/TA ratio than both the organic and inorganic nonbacterized controls (Table 5). Two-way factorial analysis showed no PGPR treatment effect and no interaction between factors and treatments. Hence, all treatments were combined by the factors. Organic fertilized tomato plants had significantly higher TSS/TA ratio than inorganic fertilized plants ($P = 0.0029$).

PGPR effect on vitamin C content of tomato fruit.

Tomato is an excellent source of vitamin C (ascorbic acid), one of the major water-soluble antioxidants in fruits and vegetables. Vitamin C has two main forms, ascorbic acid (AA) and dehydroascorbic acid (DHA). In tomato, AA is the major form of

vitamin C. In our experiment, the organic nonbacterized control had significantly higher vitamin C content compared to the inorganic nonbacterized control (Table 6). Two-way factorial analysis showed no PGPR treatment effect and no interaction between factors and treatments. Hence, all treatments were combined by the factors. Organic fertilized tomato plants had significantly higher AA content than inorganic fertilized plants (P = 0.0016). There was no significant difference of DHA and total ascorbic acid (TAA) among all the treatments.

DISCUSSION

Our results suggest that PGPR can reduce stresses of transplant shock and reduced nitrogen availability. Transplant shock was evident in nonbacterized controls under both fertilizer regimes by very limited growth of new leaves 2 WAT (Figure 1). At this same time, plants treated with all three PGPR products had substantial new growth of leaves and taller stems which resulted in increased plant growth indices (Tables 1 and 2). Reduced transplant shock is important because plants that begin active growth immediately upon transplanting would be less susceptible to root-infecting pathogens.

The observation that nonbacterized plants supplied with organic fertilizer had mild nitrogen deficiency symptoms and reduced growth indices compared to nonbacterized plants supplied with inorganic fertilizer indicates that plants in the organic fertilizer block were under mild nitrogen stress (Tables 1 and 2). The rate of organic fertilizer was calibrated to deliver the same total N/acre as the inorganic fertilizer, so it is most likely that some of the nitrogen in the organic fertilizer was more slowly released and made available to plants than was the inorganic fertilizer. Interestingly, when any of

the three PGPR products were combined with organic fertilizer, the resulting growth indices were significantly higher than those of the inorganic nonbacterized controls at both locations (Tables 1 and 2). Hence, PGPR facilitated uptake of nitrogen from organic fertilizer, thereby ameliorating the nutrient stress. One possible explanation for this observation is that PGPR treatment resulted in greater microbial activity in the rhizosphere, and that this enhanced release of the nitrogen from the organic fertilizer.

In both field trials, inorganic fertilized tomato plants had significantly higher yield than organic fertilized plants (Tables 3 and 4). However, there were no significant effects of PGPR products on yield, which was unexpected because the products had consistently increased the growth of plants through the season. One possible explanation is that the increases in vegetative growth caused by PGPR were at the expense of fruit development, although this has not been found to be a common result in previous field work with PGPR.

Effects of PGPR and fertility regime on fruit quality were determined by measuring sugar content (total soluble solids), acidity, and vitamin C content. Our results showed that PGPR can affect fruit quality with organic fertilizer. Two PGPR products – AgBlend and BioYield – increased flavor as indicated by a significant increase in TSS/TA (Table 5). Other components of quality were not affected by PGPR.

Fruit quality was also affected by the fertility regime used. With organic fertilizer, significant increases were noted across all treatments for TSS, TSS/TA, and AA compared to the inorganic fertilizer (Tables 5 and 6). Although marketable yield was less with organic fertilizer, fruit quality was higher. Moreover, some PGPR in combination with organic fertilizer may improve tomato flavor quality and nutrient quality.

Table 1. Plant growth index[a] over time at Sand Mountain Research and Extension Center in Crossville, AL.

Treatment		2 WAT	4 WAT	7 WAT
Fertilizer	PGPR			
Organic	AgBlend	54.38[bc]	296.9[a]	531.4[a]
	BioYield	59.92[ab]	277.8[a]	491.5[b]
	Equity	50.83[c]	288.5[ab]	522.4[a]
	Control	29.29[d]	185.1[e]	377.1[d]
Inorganic	AgBlend	63.29[a]	263.7[c]	533.9[a]
	BioYield	63.21[a]	255.7[bc]	536.7[a]
	Equity	52.42[c]	252.1[c]	544.9[a]
	Control	35.63[d]	229.6[d]	449.6[c]
LSD ($P = 0.05$)		6.48	19.9	26.2

[a] Plant growth index is plant height (inches) x lateral spread of the plant (inches). Values are means of 6 replications, each with four plants. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$.

Table 2. Plant growth index[a] over time at North Alabama Horticulture Research Center in Cullman, AL.

Treatment		2 WAT	4 WAT	7 WAT
Fertilizer	PGPR			
Organic	AgBlend	57.04[b]	293.2[b]	585.4[c]
	BioYield	52.21[b]	274.7[bc]	572.7[c]
	Equity	54.38[b]	275.5[bc]	563.6[c]
	Control	32.46[d]	210.7[d]	409.3[e]
Inorganic	AgBlend	67.13[a]	349.4[a]	669.3[a]
	BioYield	70.58[a]	341.6[a]	658.3[ab]
	Equity	70.29[a]	329.3[a]	626.9[b]
	Control	41.92[c]	262.5[c]	497.6[d]
LSD ($P = 0.05$)		6.48	20.5	35.9

[a] Plant growth index is plant height (inches) x lateral spread of the plant (inches). Values are means of 6 replications, each with four plants. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$.

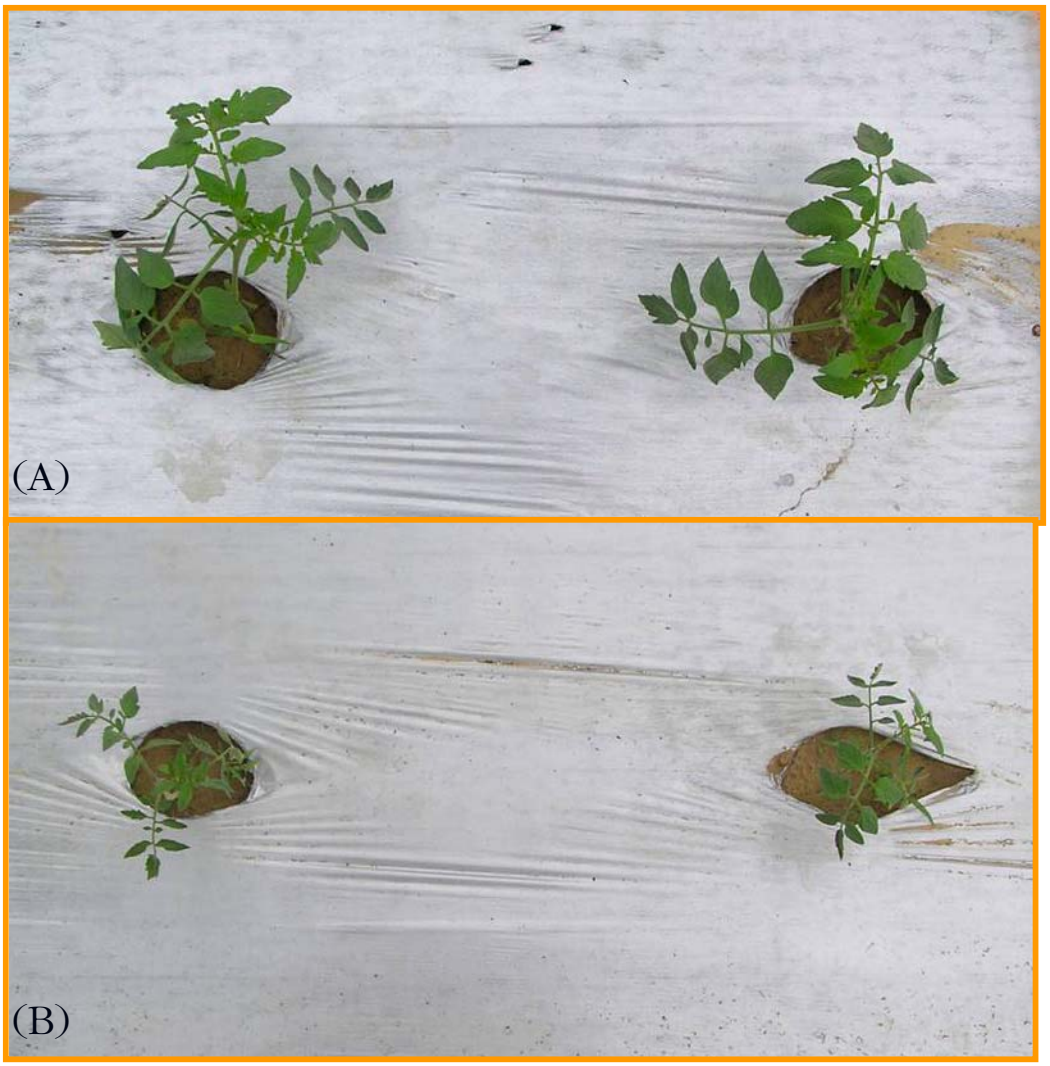


Figure 1. Reduced transplant shock by PGPR at two weeks after transplanting. (A) Ag Blend with organic fertilizer. (B) Water control with organic fertilizer.

Table 3. Tomato marketable yield at Sand Mountain Research and Extension Center in Crossville, AL.

Treatment		Total marketable weight (lbs.)	Total no. of marketable fruit	Marketable weight per fruit
Fertilizer	PGPR			
Organic	AgBlend	69.10	150.00	0.47[b]
	BioYield	72.86	147.17	0.49[ab]
	Equity	74.23	155.17	0.48[ab]
	Control	68.79	146.67	0.47[b]
Inorganic	AgBlend	74.37	153.83	0.49[ab]
	BioYield	81.12	161.33	0.51[ab]
	Equity	81.19	160.83	0.51[ab]
	Control	76.85	149.17	0.52[a]
LSD ($P = 0.05$)		16.13	32.24	0.04

Values are means of 6 replications, each with 10 plants. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$.

Table 4. Tomato marketable yield at North Alabama Horticulture Research Center in Cullman, AL.

Treatment		Total marketable weight (lbs.)	Total no. of marketable fruit	Marketable weight per fruit
Fertilizer	PGPR			
Organic	AgBlend	67.17^{abc}	129.80^{ab}	0.52^{ab}
	BioYield	59.68^{c}	117.40^{b}	0.51^{ab}
	Equity	56.75^{c}	116.50^{b}	0.49^{b}
	Control	65.01^{bc}	130.33^{ab}	0.49^{b}
Inorganic	AgBlend	77.70^{ab}	142.40^{ab}	0.54^{a}
	BioYield	78.03^{ab}	142.60^{ab}	0.55^{a}
	Equity	76.39^{ab}	139.50^{ab}	0.54^{a}
	Control	82.88^{a}	153.83^{a}	0.54^{a}
LSD (P = 0.05)		15.98	27.08	0.04

Values are means of 6 replications, each with 10 plants. Superscripted letters indicate values within the same column that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at P = 0.05.

Table 5. Sugar and acidity composition in tomato fruit.

Treatment		TSS[a] (%) (Brix)	pH	TA[b] (%)	TSS/TA
Fertilizer	PGPR				
Organic	AgBlend	1.20[a]	5.744	0.0037	325.71[a]
	BioYield	1.10[ab]	5.763	0.0035	320.83[a]
	Equity	1.14[ab]	5.736	0.0037	311.19[ab]
	Control	1.04[abc]	5.744	0.0040	260.00[bc]
Mean of all PGPR treatments		1.12*	5.748	0.0037	305.22*
Inorganic	AgBlend	1.04[abc]	5.672	0.0040	260.00[bc]
	BioYield	0.88[c]	5.758	0.0038	235.12[c]
	Equity	1.10[ab]	5.674	0.0040	276.75[abc]
	Control	0.98[bc]	5.700	0.0039	254.37[bc]
Mean of all PGPR treatments		1.00	5.704	0.0039	255.54
LSD ($P = 0.05$)		0.20	0.150	0.0005	58.24

[a] TSS = Total soluble solids. [b] TA = titratable acidity (as citric acid %).

Values are means of 12 replications. Superscripted letters indicate values within the same column (except for the mean of all PGPR treatments) that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$.

* Indicates a significant increase compared to inorganic fertilizer using Fisher's LSD test at $P = 0.05$.

Table 6. Vitamin C content[a] in tomato fruit.

Treatment		AA	DHA	TAA
Fertilizer	PGPR			
Organic	AgBlend	13.12[ab]	5.31	18.43
	BioYield	12.52[abc]	4.25	16.78
	Equity	13.47[ab]	3.35	16.82
	Control	13.74[a]	4.73	18.47
Mean of all PGPR treatments		13.18*	4.41	17.58
Inorganic	AgBlend	10.98[abc]	7.34	18.32
	BioYield	11.45[abc]	5.02	16.47
	Equity	10.93[bc]	5.01	15.94
	Control	9.79[c]	6.21	16.00
Mean of all PGPR treatments		10.82	5.85	16.67
LSD ($P = 0.05$)		2.79	6.42	6.37

[a] AA = Ascorbic acid, DHA = Dehydroascorbic acid, and TAA = Total ascorbic acid (AA + DHA). Unit = mg / 100 g fresh weight.

Values are means of 18 replications. Superscripted letters indicate values within the same column (except for the mean of all PGPR treatments) that are either significantly different (when the letters are different) or not (when the letters are the same) using Fisher's LSD test at $P = 0.05$.

* Indicates a significant increase compared to inorganic fertilizer using Fisher's LSD test at $P = 0.05$.

VI. LITERATURE CITED

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