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A Two-Strain Mixture of Rhizobacteria Elicits Induction of Systemic Resistance Against *Pseudomonas syringae* and *Cucumber Mosaic Virus* Coupled to Promotion of Plant Growth on *Arabidopsis thaliana*

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Abstract We evaluated a commercial biopreparation of plant growth-promoting rhizobacteria (PGPR) strains *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a formulated with the carrier chitosan (BioYield) for its capacity to elicit growth promotion and induced systemic resistance against infection by *Cucumber Mosaic Virus* (CMV) and *Pseudomonas syringae* pv. tomato DC3000 in *Arabidopsis thaliana*. The biopreparation promoted plant growth of *Arabidopsis* hormonal mutants, which included auxin, gibberellic acid, ethylene, jasmonate, salicylic acid, and brassinosteroid insensitive lines as well as each wild-type. The biopreparation protected plants against CMV based on disease severity in wild-type plants. However, virus titre was not lower in control plants and those treated with biopreparation, suggesting that the biopreparation induced tolerance rather than resistance against CMV. Interestingly, the biopreparation induced resistance against CMV in NahG plants, as evidenced by both reduced disease severity and virus titer. The biopreparation also elicited induced resistance against *P. syringae* pv. tomato in the wild-type but not in NahG transgenic plants, which degrade endogenous salicylic acid, indicating the involvement of salicylic acid signaling. Our results indicate that some PGPR strains can elicit plant growth promotion by mechanisms that are different from known hormonal signaling pathways. In addition, the mechanism for elicitation of induced resistance by PGPR may be pathogen-dependent. Collectively, the two-Bacilli strain mixture can be utilized as a biological inoculant for both protection of plant against bacterial and viral pathogens and enhancement of plant growth.

Key words: Induced systemic resistance, plant growth promotion, *Arabidopsis*, PGPR

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Most approaches for using plant growth-promoting rhizobacteria (PGPR) have relied on application of single bacterial strains. This may partially account for the reported inconsistent performance by PGPR in the field, because a single biological agent is not likely to be active in all soil environments. In contrast, when mixture treatments of PGPR strains were applied directly to seeds or seedlings before sowing or transplanting, mixture treatment of PGPR improved the effect of plant growth promotion or induced systemic resistance in many cases, compared with single treatment [6, 7, 19]. To improve the capacity of PGPR on plant growth promotion and biological control, many studies have been performed. Recently, a biological preparation, LS213, which contains industrially formulated spores of *Bacillus subtilis* strain GB03 as a growth-promoting agent, *B. amyloliquefaciens* strain IN937a as an induced resistance agent, and 2.5% chitosan as carrier, was tested for its capacity to promote growth of tomato and pepper and to control plant pathogens and nematodes in tomato and pepper under greenhouse and field conditions [8–11, 14].

In previous studies under field and greenhouse conditions, PGPR strains elicited systemic protection of pepper, tomato, or cucumber from viral, fungal, and bacterial pathogens [8, 15, 19, 29–32]. In addition, chemical triggers such as salicylic acid, β-aminobutyric acid (BABA), and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH, Actigard) have been reported as strong inducers of plant defense [26, 28]. In many cases, the application of these chemical triggers resulted in reduced plant growth [1, 5]. For example, BTH significantly decreased wheat growth under limited nitrogen conditions in the greenhouse and field [5]. The results indicated that reduction of plant growth after triggers of induced resistance can be explained as allocation fitness cost in the limited resource condition

of nutrient or minerals for growth and plant defense against pathogen attack [4, 5]. Beside the chemical triggers, PGPR elicited induced resistance, referred to as induced systemic resistance (ISR), as well as plant growth promotion. Previous studies have not considered the mechanisms of PGPR when growth promotion and ISR are elicited at the same time.

In this study, we evaluated whether a PGPR formulation would elicit plant growth promotion, by using *Arabidopsis thaliana* to test signaling pathways with different hormonal mutants such as auxin, gibberellic acid, ethylene, jasmonate, salicylic acid, and brassinosteroid insensitive lines. Furthermore, the biopreparation was also evaluated for ISR against a bacterial pathogen, *Pseudomonas syringae* pv. tomato, and a viral pathogen, *Cucumber Mosaic Virus*, with a focus on SA signaling in *Arabidopsis thaliana*. To our knowledge, this is the first report to elucidate signaling pathways in plant growth promotion and induced resistance elicited by a PGPR-based biopreparation in *A. thaliana*.

MATERIALS AND METHODS

Biopreparation Treatments

A biopreparation preparation, which consisted of industrially formulated endospores of *Bacillus subtilis* strain GB03 and *B. amyloliquefaciens* strain IN937a and chitosan as the formulation carrier, was used in this experiment. The PGPR/chitosan formulation referred to as biopreparation (= LS213) or BioYield was supplied by Gustafson, Inc. (Plano, TX, U.S.A.) [9]. The biopreparation was mixed into peat-based soilless growth media (Speedling Inc., Bushnell, FL, U.S.A.) at a ratio of 1:40 (v/v). Each *Arabidopsis* seed was sown directly into the amended soilless growth medium and maintained in a temperature-controlled greenhouse at the Plant Science Research Center at Auburn University, Alabama, U.S.A. Ambient air

temperatures in the greenhouse were maintained at 25°C day/21°C night throughout the year. Watering procedures were carried out routinely by greenhouse personnel with no application of fertilization. Treatments consisted of biopreparation and a nonbacterized control. Preliminary experiments that included a control treatment consisting of chitosan without the addition of PGPR did not result in the enhanced growth when the treatment included PGPR (Table 1). Therefore, a chitosan-alone treatment was not included in further experiments.

Arabidopsis Lines and Preparation

All mutant and transgenic lines were derived from parental *A. thaliana* ecotypes Col-0 or Wassilewskija (WS), which were obtained from the Ohio State University Stock Center (Columbus, OH, U.S.A.). Mutant lines tested in this study were described in the previous study [21, 22, 25]. The background of *gai2* and *eir1-3* is WS *cbb1* is C24 and that of the other mutants is Col-0. Seeds were surface-sterilized with 70% ethanol for 1 to 2 min followed by treatment with 1% sodium hypochlorite for 20 min prior to planting in the biopreparation-treated soilless medium in a greenhouse. A stock solution of benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Syngenta, Research Triangle Park, NC, U.S.A.) at 0.33 mM was freshly prepared in sterile distilled water for each experiment and ammended around *Arabidopsis* seedlings at the same time as the biopreparation application. BTH, a chemical trigger, was used as a positive control.

Population Dynamics of PGPR Strains on the Root of *Arabidopsis*

To evaluate the population dynamics of strains GB03 and IN937a after application into the soilless medium, spontaneous rifampicin-resistant mutants of each strain were screened by growing colonies on TSA amended with 100 µg/ml rifampicin (rif-TSA) [21, 25]. Isolated colonies with growth

Table 1. Effect of biopreparation and chitosan on plant growth promotion and induced systemic resistance in *Arabidopsis*.

Treatments[a]	Plant growth[b]		ISR	
	Foliar fresh weight (mg)	Statistic significance (P=0.05)	CMV[c]	*P. syringae* pv. tomato[d]
Biopreparation	568	*	*	*
Chitosan	249	–	–	–
BTH	NT	NT	*	*
Control	268	–	–	–

Numbers represent mean of 10 replications per treatment, one seedling per replication.
[a]Biopreparation was mixed into soilless medium in a square pot and 10 seeds of *Arabidopsis thaliana* ecotype Col-0 were seeded in each row in the pot.
[b]Growth promotion by biopreparation was assessed on different mutant lines of *Arabidopsis* 5 weeks after seeding described in Materials and Methods. NT=not tested.
[c]CMV was challenged on 3 leaves of 3-week-old *Arabidopsis* seedlings per plant mechanically. Disease severity was measured two weeks after inoculation by disease index described in Materials and Methods.
[d]*P. syringae* pv. tomato DC3000 was challenged on leaves by spraying. The disease severity was measured by recording the percent of total plant leaf surface showing symptoms for each plant.
*Indicates significant difference compared with control on Fisher's LSD test at P=0.05.

rates similar to the wild-type strains were stabilized by growing on rif-TSA for several generations. The rif-mutants of each strain were applied to *Arabidopsis* seedlings in the potting media as described previously [21, 25]. At 0, 3, 4, and 5 weeks after treatment with rif-resistant PGPR strains, root systems on each plant were taken and ground with a sterile mortar and a pestle. The dilution plating method was used to isolate rif-resistant colonies on TSA amended with 100 μg/ml rif for selection of rif resistance and 100 μg/ml cyclohexamide for inhibition of fungal growth. The colony-forming units (CFU) were counted 48 h after incubation at 27°C. To evaluate plant growth promotion, the total leaf surface area of each *Arabidopsis* plant treated with the biopreparation was measured five weeks after seeding as described in Ryu *et al.* [21, 23]. Water application was used as the control.

Cucumber Mosaic Virus Inoculations and Enzyme-Linked Immunosorbent Assay (ELISA)

Preparation and inoculation of CMV were performed as described by Ryu *et al.* [22]. CMV in *Arabidopsis* tissue was detected by antigen-coated plate, indirect ELISA, as described by Garcia-Ruiz and Murphy [2].

Assay of ISR Against *P. syringae* pv. tomato

Inoculation of *Pseudomonas syringae* pv. tomato DC3000 was performed as described by Ryu *et al.* [21]. Two weeks after seeding, one seedling of Col-0 was transplanted into a 10-cm square pot mixed with the biopreparation. Two weeks after transplanting, freshly prepared suspensions of *P. syringae* pv. tomato in sterile containing 200 μl/l Tween 20 (Sigma, St. Louis, MO, U.S.A.) were sprayed onto the leaves. Inoculated plants were placed in a dew chamber (100% humidity) under darkness for one day at 27°C and were then transferred to the greenhouse. Seven days after pathogen challenge, disease severity was measured. The "percentage disease" was measured by recording the percent of total plant leaf surface showing symptoms for each plant (0%=no symptom to 100%=most severe with necrotic symptoms), as described previously [21]. The experiment was conducted three times. BTH, a chemical inducer, was used as a positive control.

Data Analysis

Data were subjected to analysis of variance using JMP software (SAS Institute Inc., Cary, NC, U.S.A.). Significance of biopreparation treatment effects was determined by the magnitude of the *F* value at *P* = 0.05. When a significant *F* value was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD) at *P* = 0.05. Results of repeated trials of each experiment outlined above were similar. Hence, one representative trial of each experiment is reported in the results section.

Results

Preliminary Test for Effect of the Biopreparation and Chitosan on Plant Growth and Induced Resistance

To evaluate the effect of chitosan as carrier of endospores of strains GB03 and IN937a, we examined plant growth promotion and induced systemic resistance (ISR) against *Cucumber Mosaic Virus* and *Pseudomonas syringae* pv. tomato DC3000 in the soilless growth medium. First, we excluded the possibility that a bacteria carrier affected plant growth and ISR against CMV and *P. syringae* pv. tomato DC3000. Soil application of biopreparation significantly (*P* = 0.05) increased plant growth five weeks after sowing *Arabidopsis* seeds, compared with chitosan and control treatment (Table 1). Soil amendment of flak chitosan did not affect plant growth, suggesting that the plant growth promotion by biopreparation was caused by PGPR strains GB03 and IN937a. Furthermore, ISR elicited by biopreparation and chitosan was assessed against a viral pathogen, CMV, and a bacterial pathogen, *P. syringae* pv. tomato. Transplanting of *Arabidopsis* seedlings into biopreparation-amended soil elicited ISR against both CMV and *P. syringae* pv. tomato. In contrast, chitosan treatment did not show any effect against the two pathogens, compared with control. However, soil drench of 0.33 mM BTH as a positive control did reduce symptom development by CMV or *P. syringae* pv. tomato (Table 1). In this experiment, disease severity was measured by disease index (0−100) as previously described [21]. Collectively, chitosan did not affect plant growth and induced resistance against CMV and *P. syringae* pv. tomato. Thus, we used only biopreparation and control in further experiments.

Plant Growth Promotion by Biopreparation of Different Plant Hormonal *Arabidopsis* Mutants and Its Wild-Types

To elucidate the mechanisms of plant growth promotion by the biopreparation, different plant hormone mutants were used following soil application of the biopreparation. Eight *Arabidopsis* mutants including auxin/cytokinin, jasmonate, salicylic acid, gibberellic acid, and brassinosteroid-insensitive *Arabidopsis* mutant and its wild-type lines were assessed for growth promotion induced by the biopreparation. Surprisingly, mixing of the biopreparation into soilless medium increased plant growth of all mutant and wild-type *Arabidopsis* lines compared with the control, indicating that growth promotion of *Arabidopsis* elicited by the biopreparation was not dependent on signaling of the hormones tested (Table 2). Soil application of the biopreparation on wild-types Col-0, WS, and C24 increased total leaf surface area by 2.6, 1.9, and 1.6 times of water control treatment. In the control treatment, growth of *cpr1*, *gai2*, and *eir1-3* mutants was decreased relative to each background (wild-type). In contrast, growth of *ein2*, *NahG*, and *gai2 Arabidopsis* lines grown in the biopreparation-amended soil showed retarded

Table 2. Growth promotion response of *A. thaliana* mutants planted with biopreparation.

Arabidopsis lines[a]	Relevant description[b]	Total leaf surface area (cm^2)	
		Biopreparation	Control
Col-0	Wild-type	5.13*	1.98
ein2	Ethylene/cytokinin insensitive	3.78*	1.57
coi1	Jasmonic acid insensitive	4.57*	1.34
npr1	Lacks expression of PR proteins	4.18*	2.20
cpr1	Constitutive expression of PR proteins	4.12*	1.08
NahG	Salicylic acid degrading	3.82*	2.00
WS	Wild-type	5.12*	2.65
gai2	Gibberellic acid insensitive	3.08*	1.35
eir1-3	Auxin insensitive	4.39*	0.92
C24	Wild-type	4.56*	2.82
cbb1	Brassinosteroid insensitive	7.00*	1.53

Numbers represent mean of 12 replications per treatment, one seedling per replication.
[a]Biopreparation was mixed into soilless medium in a square pot and 10–15 seeds of *Arabidopsis thaliana* ecotype Col-0 were seeded in each row in the pot.
[b]Growth promotion by biopreparation was measured as total leaf surface area of different mutant lines of *Arabidopsis* 5 weeks after seeding. Col-0, wild-type ecotype Columbia; *gai2*, GA insensitive; *pid9* and *eir1-3*, auxin insensitive; *cbb1*, brassinosteroid insensitive; *ein2*, ethylene insensitive; *coi1*, jasmonic acid instensitive; NahG, nahG (SA dehydrase gene) transgenic; *npr1*, non-expression of PR protein; and *cpr1*, constitutive expression of PR protein.
*Indicates significant difference compared with control on Fisher's LSD test at $P = 0.05$.

growth, compared with each wild-type. The difference of plant growth between biopreparation and control treatments was obvious in the greenhouse or growth room. Regarding each wild-type background, biopreparation treatments on *cpr1*, *eir1-3*, and *cbb1* plants promoted greater plant growth as 3.8, 4.8, and 4.6 times than each water control treatment (Table 2). Our results indicate that any hormonal signaling pathway was not involved in growth promotion elicited by the biopreparation.

Dynamics of Bacterial Population in the Soil

A mixture of endospores of the spontaneous rifampicin mutants of strains GB03 and IN937a was attached to chitosan flakes following the procedure recommended by the manufacturer. The prepared strains GB03 and IN937a were applied into soil and populations were sustained up to 10^6 CFU/g root until four weeks after biopreparation treatment. By that time, the bacterial populations of the two strains was not different statistically. However, at five weeks after treatment, the population of strain GB03 had significantly declined upto 4×10^3 CFU/g root (Fig. 1). At the same time, plant growth on biopreparation and control was significantly different (Table 2). We measured each population density of strains GB03 and IN937a by counting each colony with distinct morphology: colonies showed yellowish and non-shiny for GB03, and milky and shiny for IN937a.

Induced Systemic Resistance Against *Cucumber Mosaic Virus*

Soil amendment of the biopreparation resulted in reduction of visual symptom development, but did not show any

effect on the virus titer in Col-0 *Arabidopsis*. Soil drench of BTH on the *Arabidopsis* significantly reduced both the disease symptoms and ELISA value. In NahG plants, both biopreparation and BTH treated *Arabidopsis* showed decreased symptom development along with virus titer (Fig. 2).

Induced Systemic Resistance Against *P. syringae* pv. tomato

To estimate ISR capacity of the biopreparation against a bacterial pathogen, an *Arabidopsis*-*P. syringae* pv. tomato pathosystem was used. Biopreparation and BTH treatments
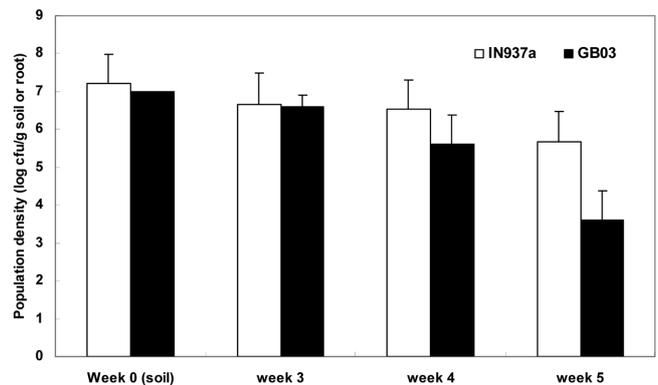
Fig. 1. Population dynamics of two rhizobacteria on the soil and rhizoplane.
The mixed rif-mutants of strains GB03 and IN937a on the chitosan flake were applied to *Arabidopsis* seedlings in the soilless media. At 0, 3, 4, and 5 weeks after treatment with rif-resistant PGPR strains, bacterial population was measured on the soil or root systems by the dilution-plating method.
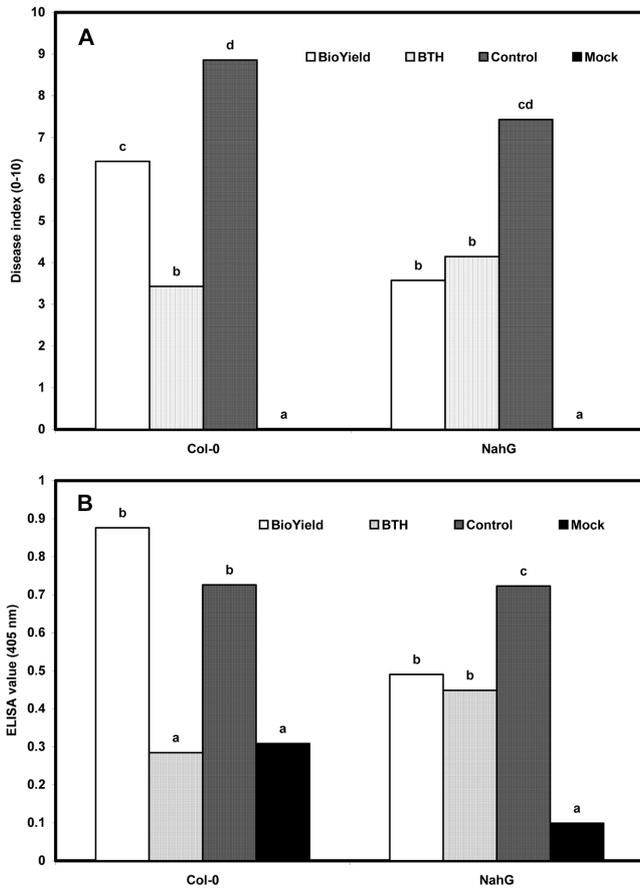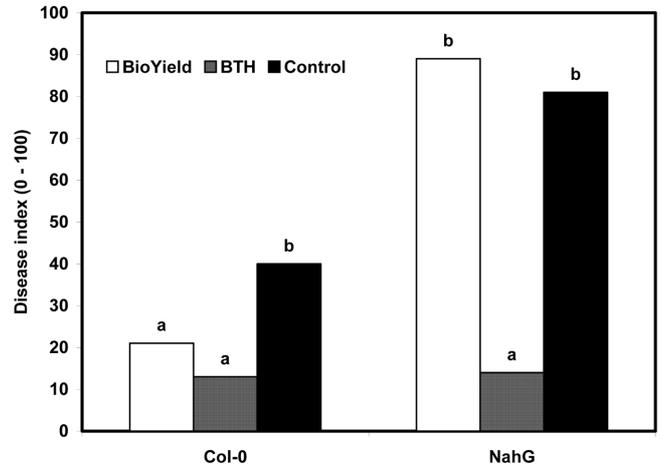
Fig. 2. Induced resistance on *Arabidopsis thaliana* against *Cucumber Mosaic Virus* by biopreparation.
Numbers represent mean of 10 replications per treatment, one seedling per replication. Biopreparation was inoculated in the soilless mixture at the same time as seeding. CMV was challenge-inoculated on three leaves per a 3-week-old seedling of *Arabidopsis thaliana* ecotype Col-0 and its NahG transgenic plant mechanically. Mock=non-virus treated control. Disease index (**A**) and viral accumulation (**B**) were measured by visual rating (0–10) and ELISA. Means followed by different letters indicate significant difference on Fisher's LSD test at $P = 0.05$.

Fig. 3. Induced resistance on *Arabidopsis thaliana* against *Pseudomonas syringae* pv. tomato DC3000 by biopreparation.
Numbers represent mean of 10 replications per treatment, one seedling per replication. Biopreparation was inoculated in the soil less mixture at the same time with seeding. *P. syringae* pv. tomato DC3000 was challenged on leaves of a 3-week-old seedling of *Arabidopsis thaliana* ecotype Col-0 and its NahG transgenic plants by spraying. The disease severity was measured by recording the percent of total plant leaf surface showing symptoms for each plant (0%=no symptom to 100%=most severe with necrotic symptoms). Means followed by different letters indicate significant difference on Fisher's LSD test at $P = 0.05$.

reduced disease severity by more than 50% compared with the control treatment in Col-0 plants (Fig. 3). However, NahG plants treated with biopreparation did not differ from control plants, whereas BTH elicited induced resistance, indicating that ISR elicited by the biopreparation required SA signaling.

Discussion

Our results presented herein suggested that a two-strain mixture of PGPR consistently enhanced growth of *Arabidopsis thaliana*. To elucidate signaling mechanisms on plant growth promotion, application of the biopreparation on the (insensitive or resistant) *Arabidopsis* mutants in several known plant hormones indicated that growth promotion

occurred independently of any known plant hormone signaling pathway: the biopreparation protected *Arabidopsis* against CMV by a pathway independent of SA signaling, but protection against *P. syringae* pv. tomato occurred by an SA-dependent pathway. Interestingly, ISR against CMV was variable, depending on measuring parameters. The virus titer of PGPR treatment was not different from that of control in Col-0 but was reduced in NahG plants in relation to control plants. However, symptom-based disease severity on both wild-type and NahG *Arabidopsis* plants was significantly reduced by PGPR application. This result agreed with previous results tested in tomato [16].

The population dynamics of two PGPR strains on the chitosan flake in the potting mixture did not statistically differ until four weeks after sowing, suggesting that initial bacterial colonization on the plant roots can play a critical role in sustaining the population. Viable bacterial cells of strain IN937a (= GB99) was greater than that of strain GB03 by 10^6 CFU/g root at five weeks after application. This result is in agreement with our previous data that the bacterial population of strain IN937a persisted more than strain GB03, when re-isolated at fall season [12]. The peat-based potting media applied to Bacilli strains GB03 and IN937a established stable populations in the root system of bell pepper that persisted throughout the growing season under field condition. More interestingly, the two-strain treatment did not affect populations of Gram-negative bacteria including beneficial indigenous rhizobacteria such as fluorescent

pseudomonads and siderophore-producing bacterial strains [12].

Many studies have been conducted to elucidate the mechanism of plant growth promotion in several crop systems [3, 13, 27]. Until now, most studies searched for bacterial determinants such as mimic plant hormones like auxin, GA, or cytokinin. Only recent studies have evaluated plant signaling in plant growth or root colonization of *Arabidopsis* by rhizobacteria [18]. Recently, Ryu *et al.* [25] reported on *Arabidopsis* hormonal mutants utilized to study plant growth promotion elicited by PGPR. In the study, ethylene seemed to play a critical role for elicitation of plant growth promotion by PGPR strains. In contrast, our present results indicate that plant growth promotion by a two-strain mixture was independent of ethylene signaling. Our results on plant growth promotion have not provided any distinct mechanisms. However, nitrogen fixation and solubilization of phosphorous by one or both of the Bacilli strains can be a good candidate mechanism that can be tested in the near future.

Questions of how PGPRs elicit both growth promotion and induced resistance at the same time have remained unanswered. To date, few researchers have conducted experimental trials to explain this phenomenon. Many chemical compounds such as SA, BABA, INA, and BTH have been reported to elicit ISR in many crops [26, 28]. Most of the previous researches on ISR concentrated only on protection of the plant against several pathogens. Despite elicitation of ISR by these compounds, grain yield was significantly reduced when a chemical inducer, Bion (= BTH), was sprayed on wheat under field condition [4], indicating that there can be a "fitness cost" to induced resistance [5]. A further study reported that *Arabidopsis* plants, which produce high levels of chitinase, peroxidase, and glucanase following application of BTH, reduced plant size and seed production related to control plants [1]. According to the concept of fitness cost for ISR, it is unclear how PGPR can sometimes elicit both ISR and growth promotion. One recent study indicated that 2,3-butanediol produced by strains GB03 and IN937a elicited both growth promotion and ISR in *Arabidopsis* [17, 24].

The use of PGPR-mediated ISR and plant growth promotion requires a delivery system that is practical on a large scale. Use of the biopreparation LS213 for preparation of transplants offers such a system. Based on the results reported herein with plant growth promotion, LS213 could be used to generate tomato transplants 1–2 weeks earlier than the typical methods used in the vegetable transplant industry, which would reduce costs of production [9].

CMV is a persistent threat to the production of many crops, particularly tomato. Because plants are especially vulnerable to CMV infection during the early growth stages, plant growth promotion of tomato at the seedling stage may provide a means to shorten this window of vulnerability.

Although treatment of tomato plants with BioYield did not protect plants from infection with CMV, compared with controls, this treatment did significantly reduce CMV-induced symptom severity and yield losses [16]. The enhanced growth of BioYield-treated tomato plants appeared to result in a form of tolerance to the infection rather than resistance. Our presented results here are agreeable with the previous study that was conducted with tomato [16].

In conclusion, our results indicate that some PGPR strains can elicit plant growth promotion by mechanisms that are different from known hormonal signaling pathways. In addition, the mechanism for elicitation of induced resistance by biopreparation can be pathogen-dependent, because protection against *P. syringae* pv. tomato was salicylic acid-dependent, whereas protection against CMV was independent of salicylic acid. Taken together, the two-Bacilli strain mixture can be utilized as a biological inoculant for both protection of plant against bacterial and viral pathogens and enhancement of plant growth.

Acknowledgments

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