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Full Length Article

Effects of *Chryseobacterium soldanellicola* T16E-39 and *Bacillus siamensis* T20E-257 on Biocontrol against Phytophthora Blight and Bacterial Wilt and Growth Promotion in Tomato Plants

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Abstract

Soil-borne diseases caused by *Phytophthora capsici* and *Ralstonia solanacearum* have threatened growth and productivity of tomato (*Solanum lycopersicum*), which is one of the most important vegetable crops in the world. It has been reported that plant growth-promoting bacteria (PGPB) enhance disease resistance and plant growth. In this study, we selected two bacterial strains to suppress soil-borne diseases caused by *P. capsici* and *R. solanacearum* in tomato plants and investigated the effects of the strains on plant growth and fruit productivity. To this end, we pre-selected 17 potential biocontrol strains out of 363 bacterial strains based on reduction of disease incidence by *P. capsici* in a radicle assay; and then through a plant assay, two strains, T16E-39 and T20E-257, were selected as biocontrol agents against *P. capsici* and *R. solanacearum* in tomato plants. We found that strains T16E-39 and T20E-257 increased plant growth; especially strain T16E-39 significantly increased fruit fresh weight in a plastic-house test and strain T20E-257 had antifungal activity against *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *P. capsici*, *Fusarium oxysporum*, *Alternaria alternata*, and *R. solanacearum*. The two selected strains had ACC deaminase and nitrogen fixation activities; produced indole-acetic acid (IAA) and siderophore, but they could not produce HCN. Based on 16S rRNA gene sequence analysis, T16E-39 and T20E-257 were identified as *Chryseobacterium soldanellicola* and *Bacillus siamensis*, respectively. These findings implied that both strains T16E-39 and T20E-257 play important roles in suppressing two soil-borne disease and inducing growth promotion; both can act as biocontrol agents and bio-fertilizer in sustainable agriculture.

Keywords: Biological control; *Phytophthora capsici*; *Ralstonia solanacearum*; Plant growth-promoting bacteria (PGPB); Tomato

Introduction

Tomato is one of the most important vegetative crops. Over 150 million metric tons of tomato are produced every year; rich source of lycopene, minerals and vitamins, including ascorbic acid and beta-carotene in tomato can play antioxidant roles in human health enhancement (Willcox *et al.* 2003; Kazemi 2014; Perez-Fons *et al.* 2014). However, tomatoes are susceptible to a variety of pathogens, and it reduced both their yield and quality constantly. (Bahramisharif and Rose 2019).

Phytophthora is one of the soil-borne and oomyceteous pathogens; capable of causing various symptoms in the whole plant; roots, stems, leaves and fruits are all susceptible to the disease (Altin *et al.* 2018). In recent decades, Phytophthora blight occurrence has increased (Lamour *et al.* 2012). Phytophthora blight in tomatoes has threatened the viability of the tomato industry (Hausbeck and Lamour 2004). Another soil-borne pathogen, *Ralstonia solanacearum* is a critical bacterial pathogen; this pathogenic bacterium causes vascular wilt disease in plants by proliferating in the vessels and blocking the translocation of water and nutrients (Eljounaidi *et al.* 2016). This induces symptoms such as wilting of terminal leaves and whole plants, cortex browning, and water soaking. *R. solanacearum* can infect almost 450 plant species belonging to 54 different plant families, even resistant cultivars (Sarkar and Chaudhuri 2016). To control these soil-borne diseases, chemical application and crop rotation protocol have been implemented. However, excessive chemical treatment can lead to environmental risks such as phytotoxicity and chemical residues (Rajkumar *et al.* 2005). For these reasons, biocontrol as an environmentally friendly approach has been studied for sustainable agricultural management (Lugtenberg and Kamilova 2009).

Free-living soil bacteria such as plant growth-promoting bacteria (PGPB) are well-known as beneficial microbes that improve plant growth and development as well as increase productivity by phytohormone production, hydrolyzing ACC, the ethylene precursor, and solubilizing diverse nutrients (Hayat *et al.* 2010). Furthermore, they can protect plants from the deleterious effects of phytopathogens by producing antagonistic substances, influencing the soil microbial community and/or inducing plant resistance (Doornbos *et al.* 2011; Beneduzi *et al.* 2012).

In this study, we i) selected bacterial strains to suppress two diseases caused by *P. capsici* and *R. solanacearum* in tomato plants and ii) determined effects of the selected strains on plant growth and fruit productivity. Furthermore, we iii) investigated bacterial characteristics related to plant growth promotion and biocontrol activities (ACC deaminase activity, IAA production, phosphate solubilization, nitrogen fixation, siderophore and HCN production), and identified the selected bacterial strains using 16s RNA gene sequence analysis.

Materials and Methods

Isolation of bacterial strains

Bacterial strains were isolated from rhizospheric soils and roots of tomato plants grown in Buyeo and Yecheon, South Korea, in 2015. For endophytic bacterial isolation, plant roots sterilized with 1% sodium hypochlorite and macerated with a sterile mortar and pestle were suspended in 0.85% NaCl. After incubation for 30 min at 28°C with shaking, each suspension was cultured on Reasoner's 2A (R2A) agar (Lab. M Ltd., U.K.) supplemented with 50 μg/mL of cycloheximide. For rhizosphere bacterial isolation, sampled rhizosphere soils were suspended in 0.85% NaCl. Cultured plates were incubated at 28°C for three days; morphologically distinct colonies were isolated and stored in vials containing R2A amended with 20% glycerol at -80°C before use.

Biocontrol activity in radicle and plant assays

Radicle and plant assays were conducted using the methods described by Chang *et al.* (2001). Briefly, for the radicle assay, tomato (cv. Juiken) seeds were germinated in Petri dishes (90 × 15 mm) with moist filter papers (Whatman No. 1) for three days at 28°C in the dark. Uniformly germinated seeds were soaked in the bacterial suspension (OD_{600} = 0.2) for three hours with shaking at 150 rpm and blotted on sterile filter papers. Ten seeds per bacterial strain were placed on 5-day-old cultures of *P. capsici* on water agar amended with 0.02% glucose; 10 m*M* $MgSO_4$-treated seeds were reserved as a control. Infected radicles were assessed three days after incubating the seeds on agar at 28°C.

For the plant assay, tomato seeds were sown in plastic pots (diameter 14 cm) containing commercial potting mixture (Bunong, Korea) and grown at room temperature in a greenhouse. Bacterial suspensions adjusted to an OD_{600} value of 0.2 with 10 m*M* $MgSO_4$ (0.5 mL/g of potting mixture) were drenched into the pots which had grown 5-week-old tomato plants. After one week, plants were inoculated by zoospore suspension of *P. capsici* (2000 zoospores/g potting mixture) at 1 cm below the soil-line near the plants, or cell suspension of *R. solanacearum* (OD_{600}=0.2, 0.25 mL/g potting mixture) were drenched. Disease incidence caused by *P. capsici* or *R. solanacearum* was assessed 5 and 7 days after inoculation, respectively and test was conducted twice again with three replications of six plants each.

Plant growth-promoting activity

To determine the growth promoting activity of bacterial strains, shoot, root and total fresh weights (FW) were measured 2 weeks after treatment of bacterial suspensions (OD_{600}=0.2), benzothiadiazole (BTH, 0.1 m*M*) and 10 m*M* $MgSO_4$ solution (untreated control) into soil (0.5 mL/g of potting mixture). Plants were taken out of pots and soil was removed by washing under running tap water. After removing the water with a towel and air-drying, fresh weights were measured. The test was conducted with 10 replicates from two experiments.

Field test

A field test with selected bacterial strains (T16E-39 and T20E-257) was conducted at the experimental farm of the National Institute of Agricultural Science, Wanju, Korea. Plants were arranged in a randomized complete block with 15 replicates per treatment. Tomato seeds were sown in a 50-hole ($5 \times 5 \times 5$ cm^3) plug tray filled with potting mixture and grown in a greenhouse. Three weeks after sowing, two bacterial suspensions, BTH (0.1 m*M*) and 10 m*M* $MgSO_4$ buffer (untreated control) were drenched into the soil (0.5 mL/g of potting mixture) twice at intervals of one week. Treated plants were transplanted into the field (30 cm between plants). For assessment of tomato productivity, mature red and yellow and unripe tomato fruits were harvested 8 weeks after transplanting, and then weighted.

Bacterial characters related to biocontrol and plant growth promotion

The antagonistic effects of two bacterial strains, T16E-39 and T20E-257, were tested against five fungal pathogens including *S. sclerotiorum*, *B. cinerea*, *P. capsici*, *F. oxysporum*, *A. alternata*, and a bacterial pathogen, *R. solanacearum*, based on growth inhibition in dual culture assays. A piece of mycelium (diameter 5 mm) from five fungal pathogens was transferred to the side of the potato dextrose agar (PDA) plate and tested bacterial suspensions (10 μL, OD_{600}=0.1) were spotted on another side. Plates were incubated at 26°C for 5–7 days, and the inhibition

zone between the bacterial strain and tested fungal pathogen was measured. To evaluate antibacterial activity against *R. solanacearum*, the selected bacterial suspensions (10 μL, OD_{600}=0.1) were dropped on Casamino acid-Peptone-Glucose (CPG) agar containing *R. solanacearum* (10^6 cfu/mL); the inhibition zone was assessed after incubation for 24 h at 30°C. Furthermore, ACC deaminase activity, phosphate solubilization, IAA production, nitrogen fixation, siderophore and HCN productions were determined as previously described (Pikovskaya 1948; Alexander and Zuberer 1991; Bric *et al.* 1991; Kremer and Souissi 2001; Penrose and Glick 2003; Pinter *et al.* 2017). *In vitro* tests were conducted with five replications from two experiments.

Bacterial identification based on 16S rRNA sequencing analysis

Bacterial genomic DNA was extracted by G-spinTM total DNA extraction kit (iNtRON, Korea) and 16S rRNA gene sequencing was performed using the universal primers fD1 and rD1 described by Weisburg *et al.* (1991). The obtained sequences were compared to 16S rRNA sequences in the EzTaxon server (http://www.ezbiocloud.net). Multiple alignments were generated using Clustal W and a phylogenetic tree was constructed following the neighbor-joining method based on 1,000 bootstrap replications using MEGA version 6.0 (Felsenstein 1985; Saitou and Nei 1987).

Statistical analysis

Statistical analysis of the data was performed using Statistical Analysis System (S.A.S.) (version 9.1.3, S.A.S. Institute Inc., Cary, N.C.). All the experiments were conducted twice and pooled data from repeated experiments were used for statistical analysis after confirmation of homogeneity of the variance by Leven's test. Percent data was analyzed statistically after arcsine square-root transformation and results of the experiment are expressed as mean ± standard error (SE). Analysis of variance was performed using the general linear model (GLM) and the means were separated by the least significant difference (LSD) test at $P < 0.05$.

Results

Biocontrol activity in radicle and plant assay

A total of 363 bacterial strains were isolated from rhizospheric soils and interior roots of tomato plants in plastic-houses at Buyeo and Yecheon in Korea in 2015. In order to screen bacterial strains for disease suppression against *P. capsici*, a radicle assay was performed. We selected 17 of the 363 strains (4.7%) based on less than 70% radicle infection compared to 100% infection of 10 m*M* $MgSO_4$-treated radicles (Fig. 1).

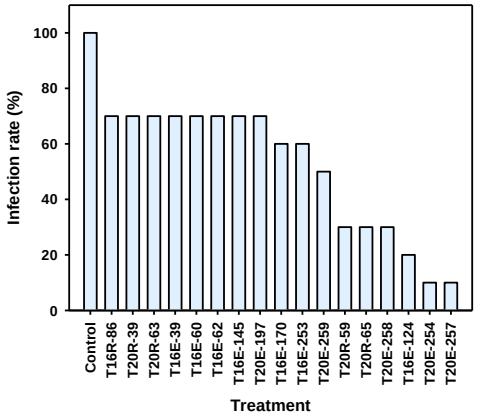

Fig. 1: Infection rate (%) by *Phytophthora capsici* in tomato radicles dip-treated with pre-selected 17 bacterial suspensions and 10 m*M* $MgSO_4$ (untreated control) on water agar amended with 0.02% glucose. Infection was assessed after incubation at 28°C for three days

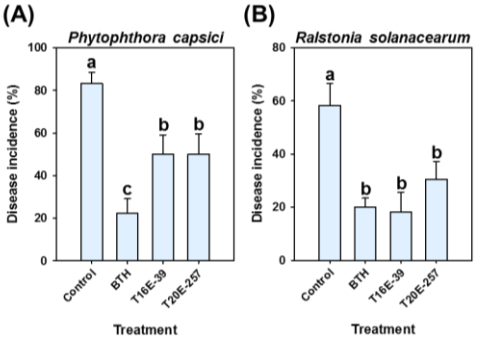

Fig. 2: Disease incidence (%) by *P. capsici* (**A**) and *R. solanacearum* (**B**) in tomato plants treated with bacterial suspensions, 0.1 m*M* BTH (a positive control) and 10 m*M* $MgSO_4$ (an untreated control). These experiments were conducted with six replications of six plants each from two experiments. Small letters on the bar mean statistical difference by the LSD test at $P < 0.05$; error bars indicate standard error

The effect of the 17 selected bacterial strains on soil borne-disease suppression was determined in 5-week-old tomato plants (Fig. 2). Treatments of T16E-39 and T20E-257 significantly reduced disease incidence by *P. capsici* compared to control plants (Fig. 2A; $P < 0.05$). Similarly, these two strains also significantly suppressed disease incidence caused by *R. solanacearum* in tomato plants (Fig. 2B; $P < 0.05$).

Plant growth-promoting activity

Fresh weights of plants were measured to determine growth promotion of the two selected bacterial strains in tomato plants (Fig. 3A). Both selected bacterial strains, T16E-39 and T20E-257, clearly increased shoot (Fig. 3B; $P < 0.05$), root (Fig. 3C; $P < 0.05$) and total fresh weights (Fig. 3D;

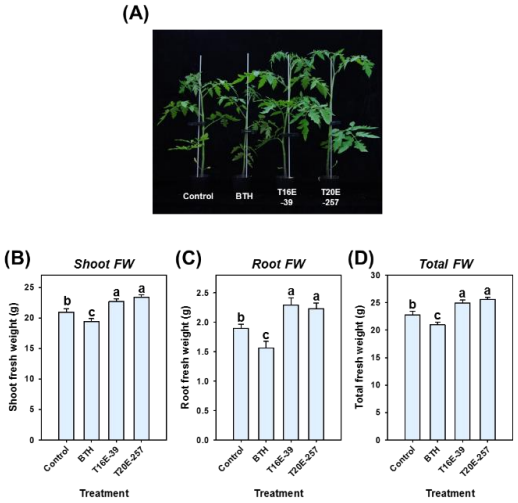

Fig. 3: Shoot (**B**), root (**C**) and total fresh weights (**D**) in tomato plants treated with bacterial suspensions (**A**), 0.1 m*M* BTH (a positive control) and 10 m*M* $MgSO_4$ (an untreated control). This experiment was conducted with 20 replications from two experiments. Small letters on the bar mean statistical difference by the LSD test at $P < 0.05$; error bars indicate standard error

$P < 0.05$) compared to control plants treated with 10 m*M* $MgSO_4$ solution.

Field test

To investigate the influence of two bacterial strains, T16E-39 and T20E-257, on tomato productivity, weights of ripe (red and yellow) and unripe (green) tomato fruits were measured in a plastic-house. Only treatment with strain T16E-39 significantly increased both fruit weight per plant ($P < 0.05$) and weight per fruit in ripe (red and yellow) tomatoes ($P < 0.05$), whereas strain T20E-257 did not affect productivity compared to control plants (Table 1). In addition, no significant difference was observed between treatments in weights of unripe (green) tomatoes.

Bacterial characters related to biocontrol and plant growth promotion

In order to determine antagonistic activities of the two selected bacterial strains against phytopathogens, a dual culture assay was conducted. Strain T20E-257 significantly inhibited fungal growth, including *S. sclerotiorum*, *B. cinerea*, *P. capsici*, *F. oxysporum* and *A. alternata* and bacterial growth of *R. solanacearum*; however, strain T16E-39 did not (Table 3). The bacterial characteristics related to enhancement of plant growth and disease resistance were also investigated. Two selected strains showed ACC deaminase and nitrogen fixation abilities and siderophore production (Table 2). Strains T16E-39 and T20E-257 also produced 19.5 and 5.0 μg/mL of IAA, respectively. However, only strain T16E-39 solubilized phosphate, and both the strains did not produce HCN.

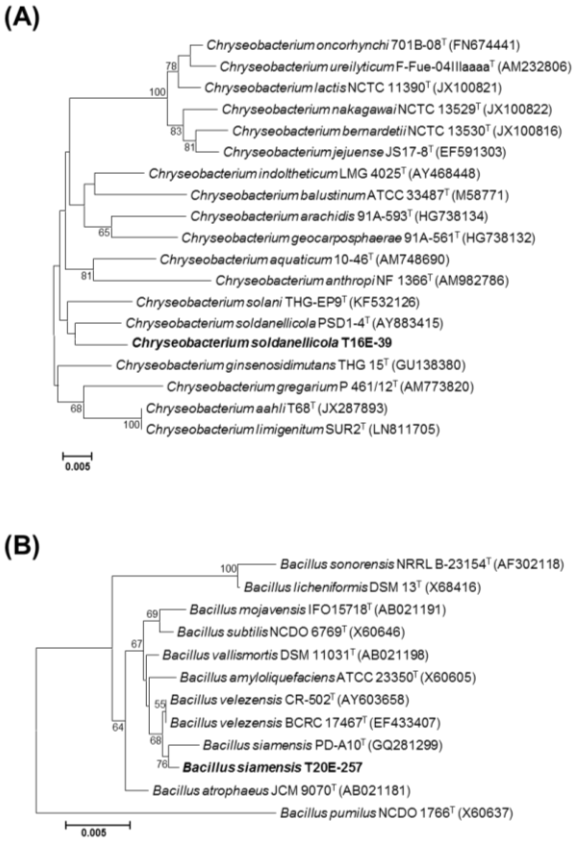

Fig. 4: Phylogenetic tree of bacterial strains T16E-39 (**A**) and T20E-257 (**B**) using neighbor-joining method based on 1,000 bootstrap replications by using MEGA version 6.0 based on the analysis of partial 16S rRNA gene sequence. Scale bars indicate the number of nucleotide substitution and letter "T" after strain name represent type strains

Bacterial identification based on 16s rRNA sequencing analysis

Strains, T16E-39 and T20E-257, were identified based on 16S rRNA sequence analysis. Phylogenetic analysis showed that T16E-39 and T20E-257 have relatedness with other bacterial strains of *Chryseobacterium* sp. and *Bacillus* sp., respectively. Strain T16E-39 was identified as *C. soldanellicola*^{T} with 98.3% identity, and T20E-257 showed 99.9% identity with *B. siamensis*^{T} (Fig. 4).

Discussion

Beneficial bacteria such as PGPB and endophytic bacteria play important roles in plant growth promotion, enhancement of resistance, and crop protection against soil-borne pathogens (Singh *et al.* 2017; Verma *et al.* 2018). For these reasons, diverse screening systems for biocontrol and growth promotion activities have been developed (Sang and Kim, 2012; Dinesh *et al.* 2015; Liu *et al.* 2017). In this study, we selected two bacterial strains out of 363 strains through radicle and plants assays; demonstrated increase of

Table 1: Fruit fresh weight in tomato plants treated with bacterial strains in a plastic-house test

Treatment	Ripe tomato fruits		Unripe tomato fruits	
	Fruit weight plant^{-1}	Fruit weight fruit^{-1}	Fruit weight plant^{-1}	Fruit weight fruit^{-1}
Control	376.7 ± 30.0 b[a]	57.6 ± 5.0 b	91.1 ± 24.4 a	43.8 ± 7.3 ab
BTH	300.0 ± 13.3 b	44.3 ± 1.9 b	80.3 ± 19.7 a	30.1 ± 11.8 b
T16E-39	512.8 ± 39.5 a	86.3 ± 11.3 a	134.2 ± 59.2 a	70.6 ± 10.6 a
T20E-257	368.5 ± 28.5 b	61.7 ± 4.8 b	96.8 ± 26.8 a	36.0 ± 6.5 b

[a] The values indicate mean ± standard errors of 15 replications and small letters mean a statistical difference at $P < 0.05$ compared with untreated control plants by LSD

Table 2: Antagonistic activity of the selected bacterial strains against phytopathogens

Strains	Inhibition zone (mm)					
	S. sclerotiorum	*B. cinerea*	*P. capsici*	*F. oxysporum*	*A. alternata*	*R. solanacearum*
T16E-39	0.00 ± 0.00[a]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T20E-257	4.85 ± 0.30	6.91 ± 0.18	1.19 ± 0.24	4.19 ± 0.12	9.24 ± 0.32	2.86 ± 0.10

[a] The values indicate mean ± standard errors of five replications from two experiments

Table 3: Plant growth-promoting traits of bacterial strains

Treatment	ACC deaminase activity	Phosphate solubilization	IAA production (μg/ml)	Nitrogen fixation	Siderophore production	HCN production
T16E-39	+[a]	-	19.53[b]	+w	++[c]	-
T20E-257	+	+	5.00	+	+	-

[a] +, indicates the presence of PGP trait, +w, indicates week presence of PGP trait and -, indicates absence of PGP trait

[b] The values indicate mean of three replications from two experiments

[c] +, presents the zone of the orange halo with < 20 mm, ++, presents the zone with more than 20 mm

growth and fruit productivity by selected strains. In addition, we assessed bacterial characteristics related to plant growth promotion and biocontrol activities and identified selected strains using 16S rRNA gene sequence analysis.

Radicle assay is known to be a reliable method for selecting antagonistic bacterial strains against *P. capsici* rapidly (Chang *et al.* 2001). In our study, 17 strains out of 363 strains suppressed infection in tomato radicles. We further tested the effects of the 17 strains on suppression of oomycetous soil-borne disease caused by *P. capsici* and bacterial soil-borne disease by *R. solanacearum* in tomato plants. Two soil-borne diseases were significantly reduced in plants treated with T16E-39 and T20E-257 compared to untreated control. These results indicated that two bacterial strains protected tomato plants from two types of soil-borne pathogens, *Phytophthora capsici* and *R. solanacearum*. Similarly, the strains *B. subtilis* and *Chryseobacterium* sp. could control Phytophthora blight and Ralstonia wilt in tomato plants (Liu *et al.* 2014).

It has been reported that treatment with beneficial bacteria induced not only plant growth promotion but also increased fruit production in diverse agricultural crops (Sang *et al.* 2013; Arif *et al.* 2016; Li *et al.* 2019). Strains T16E-39 and T20E-257 increased fresh weights (shoot, root, and total weight) of tomato plants compared to the control. However, fruit weight per plant and per ripe fruit (red and yellow tomatoes) were only increased in plants treated with T16E-39. These results implied that the two strains could improve growth promotion in tomato plants; only strain T16E-39 led to an increased rate of maturation in red and yellow tomatoes. Beneficial microbes used as bio-fertilizers could produce plant hormones such as indole acetic-acid (IAA), gibberellin (GA) and cytokinin (CK) and increase photosynthesis performance. In some cases, they elicit plant tolerance to stress and protection against plant pathogens, thereby resulting in crop enhancement (Bhardwaj *et al.* 2014). Accordingly, the two strains, T16E-39 and T20E-257, could play a role as bio-fertilizers in tomato plants by inducing growth promotion and protection against two soil-borne diseases.

Antagonistic effects could be attributed to the production of antimicrobial compounds, including antibiotics, which lead to the inhibition of protein synthesis, disruption of membrane structural integrity, and leakage of potassium ions in fungal and bacterial pathogens (Nagórska *et al.* 2007; Yuan *et al.* 2012). In our dual culture studies, strain T20E-257 had a high antagonistic ability against tested fungal and bacterial pathogens. These results indicate that this strain could reduce soil-borne diseases, including Phytophthora blight and bacterial wilt by directly inhibiting the growth of the pathogen. In contrast, strain T16E-39 did not affect the tested pathogens in a dual culture assay. Biocontrol activity is known to be induced by interactions with the host plant, biological agent, pathogens, as well as the physical and biological environments (Kumar *et al.* 2014). Therefore, we expected that T16E-39 would suppress diseases caused by *P. capsici* and *R. solanacearum* differently than strain T20E-257 in tomato plants.

PGPB has been reported to stimulate plant growth and induce resistance to soil-borne pathogens through the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase which degrades stress ethylene, ACC, a compound produced under stress conditions, and enhances plant root growth; production of IAA, one of the key phytohormones which improve nutrition uptake efficiency in roots. In addition, it has phosphate solubilization and nitrogen fixation abilities to help uptake phosphorus and nitrogen; has antagonistic ability against phytophathogenic

microbes through the production of siderophores and HCN (Patten and Glick 2002; Glick 2012; Babu *et al.* 2015; Singh and Jha 2017). Our results suggest that two selected strains could promote plant growth through degradation of ACC, providing nitrogen as nutrition (T20E-257 provided phosphorus) and phytohormone IAA. The two strains might be expected to suppress diseases using their produced siderophore or other mechanisms, but HCN is not related to their biocontrol abilities.

In 16S rRNA gene analysis, our results indicated that strain T16E-39 shows maximum similarity (98.3%) with *C. soldanellicola* and T20E-257 matched with *B. siamensis* (99.9%). Similarly, *B. siamensis* was described to produce gibberellins (GA) and increase growth of banana plants (Ambawade and Pathade, 2013). It displays antifungal activity against two pathogens, *Alternaria brassicicola* and *Colletotrichum higginsianum*, and decrease disease incidence caused by the pathogens in Chinese cabbage plants (Lee *et al.* 2018). The effects of *C. soldanellicola* on plants has not been reported so far, in our knowledge this is the first report, although several *Chryseobacterium* sp., including *C. indologenes* and *C. balustinum* promote plant growth and resistance against *P capsici* and *Pseudomonas syringae* pv. *tomato* DC3000 in pepper and Arabidopsis plants, respectively (Solano *et al.* 2008; Sang *et al.* 2018).

Conclusion

The selected bacterial strains *C. soldanellicola* T16E-39 and *B. siamensis* T20E-257 decreased the incidence of two soil-borne diseases caused by *P. capsici* and *R. solanacearum*. In addition, the two strains induced growth promotion in tomato plants and strain T16E-39 increased ripe tomato productivity in a plastic-house test. They have also biocontrol and PGP ability; strain *B. siamensis* T20E-257 directly inhibited growth of tested six phytopathogens. On the basis of these results, we expected that strains *C. soldanellicola* T16E-39 and *B. siamensis* T20E-257 could be used as biocontrol agent against *P. capsici* and *R. solanacearum* as well as bio-fertilizers in tomato plants for sustainable agriculture.

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