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Pseudomonas putida Strain 17 Isolated from Replant Soil Promotes Tomato Growth and Inhibits Conidial Germination of Soilborne Plant Pathogens

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The induction of growth promotion on numerous crops by rhizobacteria is a well documented phenomenon. In case of tomato (*Lycopersicon esculentum*), fruit yield is higher in replant soil than that in fresh soil. To investigate what kind of rhizobacterium is involved, microbial community in rhizosphere and on rhizoplane of tomato plants from each soil was analyzed by dilution plating on selective media. Many Gram-negative bacteria and actinomycetes were isolated from tomato in replant soil. One Gram-negative rhizobacterium isolated was identified as *Pseudomonas putida* based on its biochemical characteristics, fatty acid methyl ester analysis and 16S rDNA sequence. This bacterium designated strain 17 inhibited the growth of *Pseudomonas corrugata*, and increased growth of tomato seedlings. In addition, its culture filtrate inhibited conidial germination of plant-pathogenic fungi such as *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *F. oxysporum* f. sp. *cucumerinum*, and *Nectria radicicola*. Scanning electron microscopy revealed strain 17 colonized and persisted on the epidermal surfaces of tomato radicles and roots. These results suggest that *P. putida* strain 17 may serve as a biological control agent to suppress multiple soil-borne diseases for tomato plants. Increased microbial populations that suppress deleterious microorganisms including pathogens could be one of the major factors in increased tomato yield in replant soil.

Keywords : biocontrol, *Lycopersicon esculentum*, plant growth-promoting rhizobacteria, PGPR

Tomato (*Lycopersicon esculentum*) is one of the most important vegetable crops in Korea. Tomato was cultivated in 4,102 ha in 2003, and mainly (97%) in greenhouses to produce high quality of tomatoes (Ministry of Agriculture and Forestry, 2004). The cultivation in greenhouse that has limited flexibility often leads to repeated plantation of tomatoes in the same field. In addition, fortified environmental regulations have prompted application of biological control in practices.

Suppressive soils have been described for many soilborne plant pathogens and pests including fungi (Broadbent and Baker, 1974; Cook and Rovira, 1976; Mazzola and Gu, 2002; Scher and Baker, 1980), bacteria (Menzies, 1959; Shiomi et al., 1999), and nematodes (Kerry, 1988; Westphal and Becker, 1999). Some suppressive soils also provoked yield enhancements. Studies on the suppressive soils have provided extensive information on abiotic and biotic factors on microbial resources that have shown potential as biological control and plant growth-promoting agents.

Biological control for increased disease control and productivity has been reported in several crops including tomato, hot pepper, cucumber, and tobacco by a strain or mixtures of rhizobacteria stimulating plant innate defense responses (Anith et al., 2004; Jetiyanon et al., 2003; Kloepper, 1997; Kloepper et al., 2004; Murphy et al., 2003; Murphy et al., 2000; Raupach and Kloepper, 1998; Ryu et al., 2004). Interestingly, these rhizobacteria showed broad inhibitory spectrum of protection against multiple pathogens. Based on our observation that higher amounts of tomato fruits were harvested from an experimental field with continuous cultivation for 12 years, we have speculated that structural difference in microbial community might have resulted in increased yield. Therefore, this study was conducted to investigate microbial communities in fresh and replant soil of tomato, to select biological agents inhibiting soil-borne pathogens and promoting plant growth, and to identify their taxonomical positions at the biochemical and molecular levels.

Materials and Methods

Microorganisms. Pathogens used in this study were *Pseudomonas corrugata*, the causal agent of tomato pith necrosis, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, the

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causal agent of tomato crown and root rot, *F. oxysporum* f. sp. *cucumerinum*, the causal agent of cucumber wilt, and *Nectria radicicola*, the causal agent of ginseng root rot. Cell suspensions of *P. corrugata* and selected rhizobacteria were retrieved from 2-day-old cultures grown on King's B agar (Difco Lab., USA) at 28°C in the dark and grown on nutrient broth (Difco Lab.) at 28°C with 150 rpm. Conidia were harvested from 10-day-old cultures grown on potato dextrose agar (PDA; Difco Lab., USA) at 25°C in the dark. Above microorganisms and selected rhizobacteria were maintained as conidial suspension amended with 20% glycerol at −70°C until use.

Field trials. Tomato seeds (cultivar 'Momotaro') were sown in pots (9 cm diameter) filled wit a mixture of perlite and peatmoss (7:3), and grown in a greenhouse. Tomato seedlings at the fifth leaf stage were transplanted in a replant soil for 12 years and another fresh soil with the first tomato cultivation in the greenhouse. A randomized complete block design was used with three replicates per treatment. Plot size was 56 m^2 (1.4 m × 40 m) with more than 80 plants per plot. Before transplanting, fertilizers were added at the rate of 30,000 kg of compost, 200 kg of NO_3^-, 320 kg of P_2O_5, and 200 kg of K_2O and mixed thoroughly. Stem diameter, leaf length, leaf width, and fruit yield were measured 90 days after transplanting.

Analyses of microbial communities. More than 10 tomato roots were sampled from each plot of fresh and replant soil. To estimate the microbial populations on rhizoplane, soil attached to the root was carefully detached and washed with tap water. And then, 5 g of fresh root was collected. For enumeration of rhizosphere bacteria, roots and attached soils (10 g) were collected from each plot. After adding 45 ml of distilled water, all combined samples (5 g) were shaken for 30 min at 200 rpm, and 0.1 ml of aliquot was plated by 10-fold serial dilution on media. Gram-positive/-negative bacteria were enumerated on D2 medium (Kado and Heskett, 1970) and crystal violet medium (Gould et al., 1985) 2 days after incubation at 28°C. Filamentous fungi and actinomycetes were estimated on rose bengal base (Difco Lab., USA) and egg albumin agar (Corke and Chase, 1956) 3 days after incubation at 24°C.

Analyses of soil mineral, pH, and electrical conductivity. Soil samples were retrieved from three sites of each replicate. Analyses of pH, electrical conductivity (EC), contents of P, Ca, and K were performed according to the methods described in the standard manual (Rural Development Administration, 1988).

Selection of antagonistic and tomato growth-promoting rhizobacteria. To isolate beneficial rhizobacteria from tomato grown in replant soil, root samples were collected from 10 tomato plants producing good amount of fruit yield. After washing off surface soil with running tap water, the root samples were surface-sterilized with 70% ethanol and 1% sodium hypochlorite. After another washing with sterile water, the samples were macerated with 45 ml of sterilized distilled water. An aliquot (0.5 ml) from 10-fold series dilution was spread on King's B agar and kept at 28°C for 24 h. Cell suspension of *P. corrugata* at 3×10^8 CFU/ml was overlaid on the top, and the plates were incubated for additional 24 h at the same condition. Rhizobacterial colonies inhibiting growth of *P. corrugata* were selected. To select tomato growth-promoting endophytic rhizobacteria among bacterial isolates inhibiting *P. corrugata*, Tomato seedlings (cultivar 'Momotaro') were grown in plastic pot (9 cm diameter) with soils collected from replant soil and autoclaved before use in a greenhouse. Tomato seedlings at the third leaf stage were cut above cotyledon part and dipped into rhizobacterial suspensions (10^8 CFU/ml) for 2 h. Tomato seedling cuts were transplanted in sterile soil. The fresh weight of tomato plants was assessed 20 days after transplanting. Experiments were conducted independently more than three times with five replications.

Inhibition of conidial germination by culture filtrate of rhizobacteria. One of the selected rhizobacteria was inoculated into 100 ml of nutrient broth and grown for 36 h at 28°C with 150 rpm on a rotary shaker. Culture was sterilized by membrane (0.22 μm pore size) filtration. Conidia of *F. oxysporum* f. sp. *radicis-lycopersici*, *F. oxysporum* f. sp. *cucumerinum*, and *Nectria radicicola* were collected from PDA with mild scraping using sterile distilled water and washed twice by centrifugation. Conidial suspension was adjusted to 1×10^5 conidia/ml, and 45 ml of each suspension was placed onto hydrophobic surfaces of Gelbond (FMC Corp., USA) with addition of culture filtrateat 5 ml for each After incubation in a humid plastic box at room temperature for 12 h, conidial germination was estimated by considering it germinated when germ tube length was equal to or greater than spore length. Germination percentage was estimated from at least 100 conidia with three replicates per treatment. Experiments were repeated times independently.

Identification. To identify the selected rhizobacteria, carbon source utilization profiles, fatty acid compositions, and 16S rDNA sequence of bacteria were investigated as described by Jeong et al. (2003). Briefly, carbon source utilization of rhizobacteria and reference organism was analyzed using Biolog microplates (Biolog GN MicroPlate; Biolog, USA). Fatty acid methyl ester (FAME) was analyzed with the Sherlock Microbial Identification System

Version 2.11 (MIDI Inc, USA). Quantities were expressed as percentages of the total FAME. Biochemical analyses were repeated more than three times. 16S rDNA sequence was amplified via polymerase chain reaction and nucleotide sequence of amplified 1.5 kbp DNA fragment was analyzed using automated DNA sequencer.

Electron microscopy. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) was conducted to investigate association of rhizobacteria with tomato plants. For TEM, formvar-coated copper grid was floated on a drop of the bacterial suspension and negatively stained with 2% (w/v) uranyl acetate. After removal of excessive uranyl acetate, the grids were dried and examined with transmission electron microscope (JEOL 1010; JEOL Ltd, Japan). For SEM, tomato seeds and roots from 3-week-old seedlings were dipped for 1 h into cell suspensions (10^8 CFU/ml for germinated seeds and 10^6 CFU/ml for seedlings). Seeds were kept on a moistened filter paper without light, and seedlings were planted in a sterilized soil mix. Most seeds began to germinate 24 h after incubation. Each sample was harvested 1 and 21 days after treatment. Fixation, staining, dehydration, air-drying, and coating were conducted as described previously (Viret et al., 2004). The specimens were examined with scanning electron microscope (JSM-5410LV; JEOL Ltd., Japan).

Results

Effect of continuous cultivation on tomato plants. With repeated cultivation with tomato for 12 years, tomato growth was relatively poor compared to that in the fresh cultivation. Stem diameter, leaf length, and leaf width were inhibited by about 21%, 13%, and 13%, respectively (Table 1). On the other hand, yield of tomato fruits was about 14% higher in replant soil. Comparative analyses of microbial populations revealed that the number of viable micro-organisms within soils from replant soil was 4.9-fold higher than that from the fresh field (Table 2). Numbers of Gram-negatives, Gram-positives, and actinomycetes within soils from replant soil were higher than those within fresh soil. This tendency was reversed for filamentous fungi in the rhizosphere. Microbial density within rhizosphere was higher than that within rhizoplane by about 4.3 fold in the fresh soil. On the contrary, microbial density within rhizoplane was 3.2 times higher compared to that within rhizosphere in replant soil. Number of Gram-negative bacteria on rhizoplane from replant soil was 37 times higher than that from the fresh soil. Proportion of Gram-negative bacteria among total microbial communities was 8.3% in fresh soil. In replant soil, Gram-negative bacteria occupied 57.1% of total microbial communities.

No distinctive differences were detected in the comparison of soil profiles from a replant and fresh soil except

Table 1. Growth and yield of 'Momotaro' tomato cultivated in fresh and in replant soil[x]

Treatment[y]	Stem diameter (cm)	Leaf length (cm)	Leaf width (cm)	yield (g/plant)
Fresh soil	1.4 a[z]	49.4 a	45.5 a	7,725 b
Replant soil	1.1 b	42.8 b	39.5 b	8,774 a

[x]Tomato seedlings grown in the greenhouse was transplanted in fresh soil with the first tomato cultivation and repected for 12 years.
[y]More than 50 plants in each replication were evaluated for each item 90 days after transplanting.
[z]Means in a column followed by the same letter are not significantly different based on the Duncan's multiple range test at P = 0.05.

Table 2. Recovery of microbial complex from the rhizosphere and rhizoplane from tomato 'Momotaro' cultivated in fresh and replant soil

Treatment	Mean number of microorganisms ($\times 10^6$ CFU/g soil)[y]			
	Gram (−) bacteria	Gram (+) bacteria	Fungi	Actino-mycetes
Fresh soil				
Rhizosphere	5 b[z]	80 b	120 a	290 a
Rhizoplane	46 b	12 c	10 b	46 b
Replant soil				
Rhizosphere	2 b	400 a	12 b	300 a
Rhizoplane	1700 a	120 ab	80 b	360 a

[y]Rhizosphere and rhizoplane soil were recovered from more than 10 plants in each replication 90 days after transplanting.
[z]Means in a column followed by the same letter are not significantly different based on the Duncan's multiple range test at P = 0.05.

Table 3. Comparison of soil profiles between fresh and replant field

Treatments	pH[y]	Available P_2O_5 (mg/kg)	Exchangeable cations (cmol/kg)			Electrical conductivity (mS/cm)
			K_2O	CaO	MgO	
Fresh soil	6.1 a[z]	78 a	0.7 a	3.7 a	2.9 a	1.4 b
Replant soil	5.9 a	72 a	0.7 a	3.6 a	3.3 a	4.4 a

[y]Soil were recovered three sites of each replication 90 days after transplanting.
[z]Means in a column followed by the same letter are not significantlydifferent based on the Duncan's multiple range test at P = 0.05.

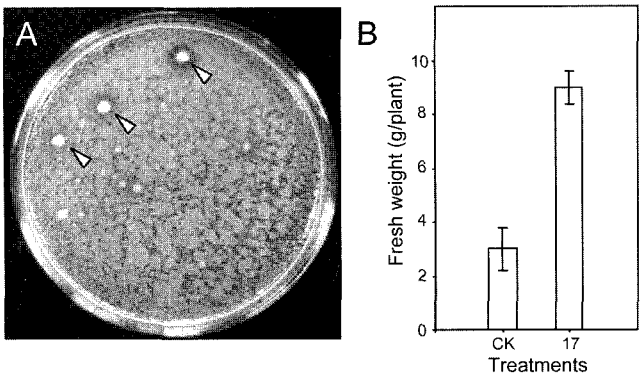

Fig. 1. Selection of rhizobacteria inhibiting tomato pathogen and promoting plant growth. (**A**) Isolation of rhizobacteria (arrows) inhibiting *Pseudomonas corrugata*. (**B**) Growth promotion of tomato plants by selected rhizobacterium, *Pseudomonas putida* strain 17.

for EC (Table 3). EC of replant soil was 3.1 times higher than that of fresh soil.

Selection of antagonistic and tomato growth-promoting rhizobacteria. Among rhizobacteria isolated from roots of tomatoes cultivated in replant soil, a total 38 isolates of Gram-negative bacteria inhibited the growth of *P. corrugata* (Fig. 1A). Among 38 isolates, strain 17 interestingly promoted tomato growth, too (Fig. 1B). Fresh weight of tomato plants treated with strain 17 was as much as 2.9 times higher than that treated with a mock sample (nutrient broth). This tendency was consistent throughout three independent experiments.

Identification of rhizobacterium. Strain 17 was rod-shaped, Gram-negative bacterium and had more than two flagella (Fig. 2). Cultural characteristics and carbon source-utilization profiles is described in Table 4. These characteristics are very similar to those of *P. putida*. The fatty acid of the strain 17 was also closely related to that of *P. putida* (Table 5). The 16S rDNA sequence showed the highest homology (98.5%) to that of *P. putida* (GenBank accesstion no. AY958233). Based on these results, strain 17 was identified as *P. putida*.

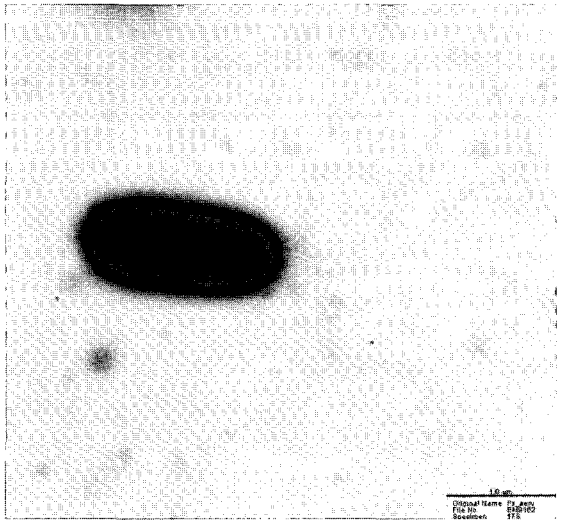

Fig. 2. Morphology of *Pseudomonas putida* strain 17.

Table 4. Biochemical tests of strain 17 compared to *Pseudomonas putida* biotype B[y]

Items	Strain 17	*P. putida* biotype B
Gram staining	–[z]	–
Oxidase	+	+
Fluorescent pigment	+	+
Arginine dehydrolase	+	+
Denitrification	–	–
Utilization		
Dextran	–	–
Tween 80	+	+
Adonitol	–	–
L-arabinose	+	+
D-fructose	+	+
L-fructose	–	–
D-galactose	+	+
D-sucrose	–	–
D-trehalose	–	–
Xylitol, turanose	–	–
D-galarutonic acid	–	+
D-glucuronic acid	–	+
D-gluconic acid	+	+
Propionic acid	+	+
D-saccharic acid	+	+
L-leucine	+	+
L-proline	+	+
L-serine	+	+
Inosine	+	+
Glycerol	+	+
Carnitine	+	+
D-sorbitol	–	–

[y] Biochemical tests were conducted using Biolog GN MicroPlate system according to the manufacturer's instructions and repeated more than three times.
[z] + indicates positive response and – indicates negative response.

Inhibition of conidial germination. Conidia of *F. oxysporum* f. sp. *radicis-lycopersici* were germinated more than 97% in sterilized distilled water at 12 h after incubation. Conidial germination was not significantly different even in 10% (v/v) of nutrient broth. However, culture filtrate of strain 17 distinctively inhibited conidial

Table 5. Fatty acid composition of total membrane lipid extracted from strain 17 and *Pseudomonas putida* biotype B[y]

Fatty acid	Strain 17	*P. putida* biotype B
10.0	0.2[z]	
10:0 3-OH	4.0	3.3
12:0	2.0	1.8
12:0 2-OH	5.4	3.6
12:1 3-OH	0.3	
12:0 3-OH	4.5	2.7
14:0	0.7	0.6
15:0	0.2	0.4
14:0 3-OH		
16:1 cis 9	30.0	26.2
16:0	36.0	31.5
17:0 cyclo	6.2	13.9
18:1 cis 11	9.2	15.4
18:1		
18:0	0.6	0.4

[y] Fatty acid methyl ester (FAME) was analyzed with the Sherlock Microbial Identification System Version 2.11 (MIDI Inc, USA). One representative trial was presented, although experiments were repeated more than three times independently.
[z] Quantities were expressed as percentages of the total FAME.

germination of *F. oxysporum* f. sp. *radicis-lycopersici* by 62%. This culture filtrate also interdicted the conidial germination of *F. oxysporum* f. sp. *cucumerinum* and *Nectria radicicola* by 52% and 97%, respectively, without significant variation throughout three independent experiments.

Root colonization of rhizobacteria. Observation using SEM demonstrated that abundant rhizobacterial cells are attached on the radicles of the germinated tomato seeds 1day after treatment (Fig. 3A and B). Only a few cells were observed on the seed coat (data not shown). Bacteria also intensively colonized around the junctions and inner part of epidermis (Fig. 3C) and produced large amount of extracellular matrix (Fig. 3D). Strain 17 still persisted on the root surface of tomato seedlings 21 days after treatment. Bacteria were colonized inside root cleavages (Fig. 3E) and asperate surface (Fig. 3F).

Discussion

The results here confirmed that continuous cultivation of tomato plants can enhance fruit yields. Based on our observation that density of rhizobacteria was relatively higher and that of filamentous fungi was lower on the rhizoplane in the continuous cultivation, continuous cultivation of tomato was suggested to be one of the major factors related with structural variation of soil microbial communities by selecting its microbial community in rhizosphere. Similar to our results, Lemanceau (1995) and Mahaffee and Kloepper (1997) demonstrated that both the rhizosphere and endorhiza select for their bacterial communities. Although total number of microorganisms was high in replant soil, diversity of microbial community was low mainly due to Gram-negative bacteria dominant in the microbial community. This result implies that continuous cultivation of tomato simplify the diversity of microbial communities. Similar observations were also made by replant of perennials as well as annual plants (Mahaffee and Kloepper, 1997; Mazzola, 1999; Mazzola et al., 2002). In this study, population of Gram-negative bacteria was dominant with 57.1% in replant soil that is similar to other studies. Populations of Gram-negative bacteria including *Pseudomonas* ranged from 43 to 76% of total rhizobacteria in tomato cultivation (Alvarez et al., 1995). Wohaka and Wolf (1994) also found that *Pseudomonas* represented mainly with 40% of total 160 bacterial strains in hydroponic system of tomato. Among these rhizobacteria, *P. fluorescens* increased significantly tomato yield by 13.3% (Gagne et al., 1993).

Analysis of soil profiles indicates that EC in replant soil is higher than that in fresh soil. Increased EC has been reported to reduce fruit size and marketable fruit yield (Dorai et al., 2001; Willumsen et al., 1996). Our result here also showed that plant growth was retarded in replant soil.

Based on above results, we hypothesized that distinctive enrichment of Gram-negative bacteria and/or a population of rhizobacteria might be one of the factors responsible for yield enhancement. Selected rhizobacterium, *P. putida* strain 17, inhibited conidial germination of multiple soil-borne plant pathogens.

One of the most important requirements of biological control agent is colonization and persistence on and in the targeted plant region (Anjaiah et al., 2003; Kim et al., 1997; Kloepper et al., 1980). Observation using SEM showed that *P. putida* strain 17 colonized successfully on the tomato root surface for up to 21 days after bacterial treatment and planting in the soil. Shapes of cell adhesion indicated that this rhizobacterium penetrated into the epidermal tissues and distributed internal cavity. Plant growth-promoting rhizobacteria often colonized in the endorhiza (Bianciotto et al., 2001; Chanway, 2000) and internal root (Kloepper, 1997). Large amount of extracellular matrix produced by strain 17 seemed helpful for cell attachment on the root surface and formation of biofilm. Production of extracellular matrix is also important for the colonization (Bianciotto et al., 2001). Our results showed that strain 17 colonized successfully on and in the tomato tissues.

In conclusion, our results suggest that increase of

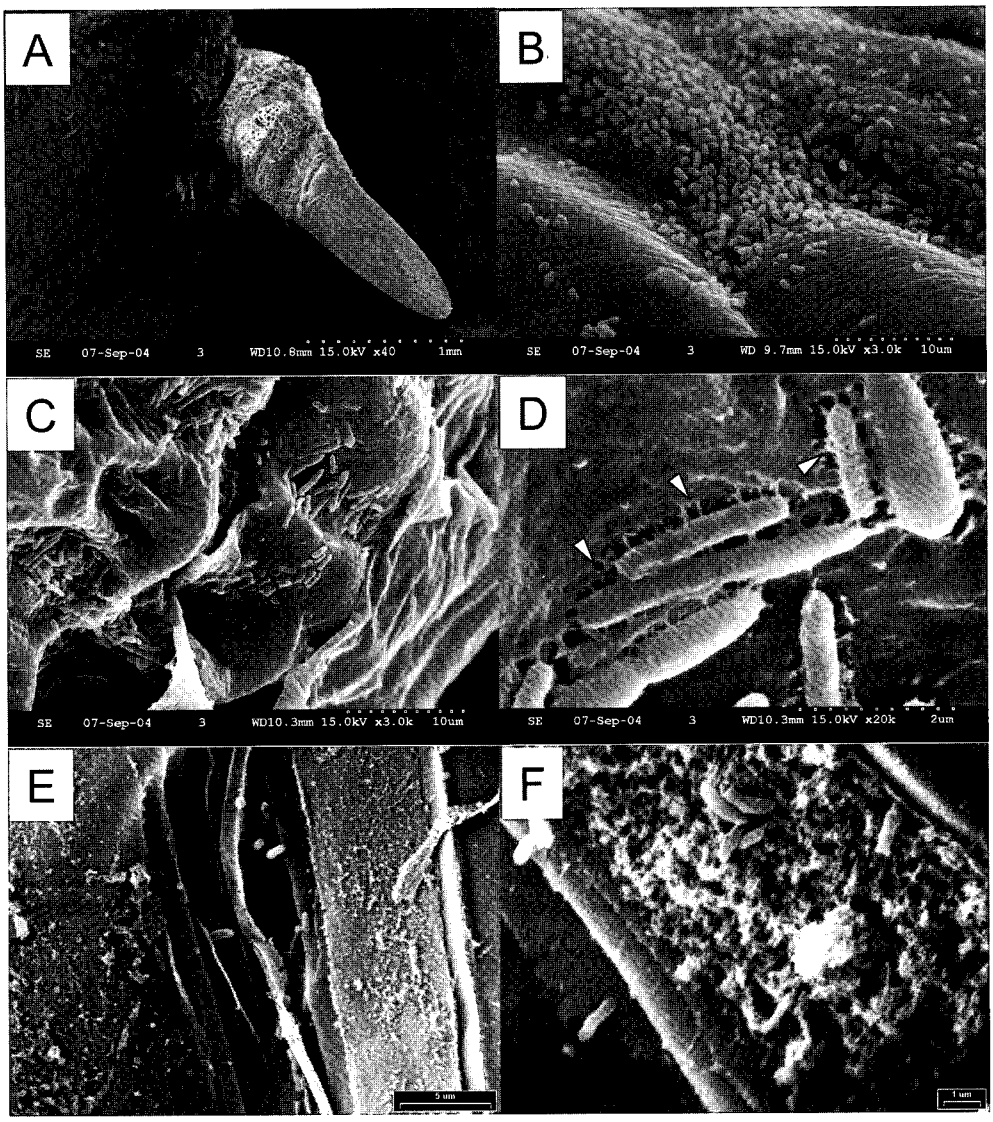

Fig. 3. Scanning electron micrograph of tomato radicle and root treated with *Pseudomonas putida* strain 17. (**A**) Tomato radicle; (**B** and **C**) Epidermal surface of radicle colonized with *P. putida* strain 17. (**D**) Extracellular matrix (arrows). of *P. putida* strain 17. (**E**) Colonization of *P. putida* strain 17 within cleavage of root surface. (**F**) Colonization of *P. putida* strain 17 on the asperate surface.

Table 6. Effects of culture filtrates from *Pseudomonas putida* strain 17 against conidial germination of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *F. oxysporum* f. sp. *cucumerinum*, and *Nectria radicicola*

Treatments	Conidial germination (%)[y]		
	F.oxysporum f. sp. *radicis-lycopersici*	*F. oxysporum* f. sp. *cucumerinum*	*N. radicicola*
Sterilized distilled water	97.6 ± 1.4 a[z]	94.3 ± 2.4 a	97.7 ± 1.6 a
Nutrient broth (10%, v/v)	96.5 ± 0.9 a	97.7 ± 2.1 a	96.9 ± 2.0 a
P. putida strain 17	36.5 ± 14.8 b	46.6 ± 24.7 b	2.5 ± 2.0 b

[y] Conidia were collected from PDA, washed twice with distilled water, and placed on the hydrophobic surface of GelBond at a final concentration of 1×10^5 conidia/ml.
[z] Means in a column followed by the same letter are not significantly different based on the Duncan's multiple range test at P = 0.05.

antagonistic microbial populations inhibiting pathogen infection and promoting plant growth may enhance tomato yield in replant soil. In addition, *P. putida* strain 17 would serve as a biocontrol agent for protection of multiple soilborne diseases and promoting tomato yield and growth enhancement.

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