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## Biocontrol of Phytophthora Blight and Anthracnose in Pepper by Sequentially Selected Antagonistic Rhizobacteria against *Phytophthora capsici*

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We previously developed a sequential screening procedure to select antagonistic bacterial strains against *Phytophthora capsici* in pepper plants. In this study, we used a modified screening procedure to select effective biocontrol strains against *P. capsici*; we evaluated the effect of selected strains on Phytophthora blight and anthracnose occurrence and fruit yield in pepper plants under field and plastic house conditions from 2007 to 2009. We selected four potential biocontrol strains (*Pseudomonas otitidis* YJR27, *P. putida* YJR92, *Tsukamurella tyrosinosolvens* YJR102, and *Novosphingobium capsulatum* YJR107) among 239 bacterial strains. In the 3-year field tests, all the selected strains significantly ( $P < 0.05$ ) reduced Phytophthora blight without influencing rhizosphere microbial populations; they showed similar or better levels of disease suppressions than in metalaxyl treatment in the 2007 and 2009 tests, but not in the 2008 test. In the 2-year plastic house tests, all the selected strains significantly ( $P < 0.05$ ) reduced anthracnose incidence in at least one of the test years, but their biocontrol activities were variable. In addition, strains YJR27, YJR92, and YJR102, in certain harvests, increased pepper fruit numbers in field tests and red fruit weights in plastic house tests. Taken together, these results indicate that the screening procedure is rapid and reliable for the selection of potential biocontrol strains against *P. capsici* in pepper plants. In addition, these selected strains exhibited biocontrol activities against anthracnose, and some of the strains showed plant growth-promotion activities on pepper fruit.

**Keywords :** antagonistic rhizobacteria, biocontrol, *Colletotrichum acutatum*, pepper, *Phytophthora capsici*

Biocontrol using microbes derived from natural sources such as soil, water, plants, and other organisms is one of the

significant strategies for ecologically sound plant disease management. Disease control by antagonistic microbes can be achieved through antibiotic production, colonization, nutrient competition, induced systemic resistance (ISR), plant growth promotion, and/or parasitism against target plant pathogens (Ahmadzadeh and Tehrani, 2009; Choudhary and Johri, 2008; Kim et al., 2012; Kloepper et al., 2004; Perazzolli et al., 2008; Sang et al., 2011; Zhang et al., 2010). Despite many attempts to develop successful biocontrol microbes (agents), these agents often do not show consistent disease suppression relative to comparable commercial fungicides. This inconsistency is often based on inappropriate screening procedures to select effective biocontrol agents, due to a lack of understanding of competition or mutualism in the niche around host plants and various environment changes. For the successful practical applications of biocontrol agents, screening procedures would need to consider interactions with potential biocontrol agents, pathogens, and host plants, thereby minimizing the labor and time needed to obtain effective biocontrol agents (Chang et al., 2001; Kim et al., 2008a; Landa et al., 2004; Maleki et al., 2011).

Pepper (*Capsicum annuum* L.) is one of the most important cash crops in the world, including in Korea; however, its production and quality have been mainly limited by plant diseases such as Phytophthora blight and anthracnose. Phytophthora blight caused by an oomycete pathogen, *Phytophthora capsici*, is a destructive disease in pepper plants. This pathogen has a wide host range with more than 50 plant species including *Cucurbitaceae*, *Leguminosae*, and *Solanaceae* (Hausbeck and Lamour, 2004; Hwang and Kim, 1995; Tian and Babadoost, 2004). This soilborne pathogen can rapidly spread to the surface water via zoospores, produce persistent propagules including sporangia, mycelia, and oospores in the debris of infected plants, and infect all parts of pepper plants such as root, crown, foliage, and fruit through water splash (French-Monar et al., 2006; Hausbeck and Lamour, 2004; Lamour and Hausbeck, 2003). For these reasons, the management of this disease has been

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considered difficult and mainly depended on the use of agricultural chemicals, which sometimes causes fungicide resistance of the pathogen and toxicity to the crops (Cohen and Coffey, 1986; Lamour and Hausbeck, 2000, 2003; Parra and Ristaino, 2001). Thus, biocontrol has been considered as an alternative strategy for disease management in this vegetable crop (Kim et al., 2008a, 2009; Sang and Kim 2011; Sang et al., 2007, 2008, 2010). In this regard, many bacterial strains belonging to the genus *Bacillus*, *Pseudomonas*, and *Paenibacillus* have been used as biocontrol microbes against various soilborne pathogens with diverse disease suppressive mechanisms as described above. Recently, an antagonistic rhizobacterial strain, *Flavobacterium johnsoniae* GSE09, was shown to produce a volatile compound (2,4-di-*tert*-butylphenol), which exhibited antioomycete activity against *P. capsici* in pepper plants (Sang and Kim, 2012a; Sang et al., 2011).

Along with Phytophthora blight, anthracnose caused by several *Colletotrichum* spp. (mainly *Colletotrichum acutatum*) is also considered as one of the most destructive diseases that limit pepper production and quality (Park and Kim, 1992; Peres et al., 2005; Sang et al., 2011). Many biocontrol microbes have been reported to be capable of reducing anthracnose occurrence on various plants including chilli pepper, cucumber, bean, and banana by either seed or foliar-spray treatment (Bardas et al., 2009; Chanchaichaovivat et al., 2008; Fu et al., 2010; Neher et al., 2009). In addition to the direct inhibitory effect of the biocontrol microbes by the spray treatment, they often show a suppressive efficacy through ISR or plant growth promotion when applied into the roots or rhizosphere. Thus, the selection of effective biocontrol microbes against these destructive diseases of pepper plants is critically important with considering their mode of action in the host-pathogen interaction.

In our previous study, we developed a sequential screening procedure to select effective biocontrol bacterial strains against *P. capsici* in pepper plants (Kim et al., 2008a). Therefore, in this study, we conducted (i) to select effective antagonistic strains against Phytophthora blight of pepper using the developed screening procedure and (ii) to evaluate the effect of the antagonistic strains selected from the screening procedure on Phytophthora blight and anthracnose occurrence and fruit yield in the plants under field and plastic house conditions from 2007 to 2009. In addition, we identified the selected bacterial strains using fatty acid-methyl ester (FAME) and 16S rRNA gene sequence analyses.

## Materials and Methods

**Isolation and preparation of bacterial strains.** Bacterial strains were isolated from the rhizosphere soil, and from the interior and surface of roots of cucumber, pepper, and

tomato plants grown in fields in two locations [Youngjongdo (37°48'N. lat., 126°49'W. long.) and Andong (36°56'N. lat., 128°76'W. long.)], Korea in 2004 and 2006. Soil-adhering plant roots were collected in polyethylene bags, stored in an ice chest, and used within 2–3 days after collection. To obtain bacterial strains from rhizosphere soil, the soil (10 g) was placed in 100 ml sterile water. For the isolation of strains from the root surface, roots (10 g) were used after the complete removal of soil. To isolate strains from the interior of root, roots (10 g) were surface-sterilized with 1% sodium hypochlorite for 90 sec, rinsed several times in sterile distilled water, and completely macerated with a sterile homogenizer. The macerated roots were placed in 100 ml sterile water and shaken at 160 rpm at 28°C for 30 min. The suspension (200 µl) was spread on tryptic soy agar (TSA) containing 50 µg/ml of cycloheximide and cultured at 28°C for 48 h. Distinct colonies appeared on TSA were selected, streaked on nutrient agar (NA), and incubated at 28°C for 48 h. From these cultures, single colonies were obtained and cultured in nutrient broth (NB) in a rotary shaking incubator (160 rpm) at 28°C for 24 h, and then stored in NB containing with 20% glycerol at –70°C until used.

For pepper radicle and seedling assays, bacterial strains were streaked on NA and incubated at 28°C for 48 h. The bacterial cells were harvested and suspended in 10 mM MgSO<sub>4</sub> solution, and then adjusted to 10<sup>8</sup> cells/ml (OD<sub>600</sub> = 0.5) with a spectrophotometer (Du® 650, Beckman Coulter, Fullerton, CA, USA). Likewise, for plant, field, and commercial plastic house tests, single colonies were inoculated in 5 ml NB and incubated in a shaking incubator (160 rpm) at 28°C for 24 h. The pre-cultured bacterial cells were further incubated in 500 ml NB in a shaking incubator (160 rpm) at 28°C for 48 h. The cells were harvested in 10 mM MgSO<sub>4</sub> solution by centrifuging at 5,000 × *g* at 20°C for 15 min, and the harvested pellets (bacterial cells) were washed twice with the same solution by centrifugation. Bacterial suspensions were adjusted to 10<sup>8</sup> cells/ml in the solution as described above.

**Radicle and seedling assays against *P. capsici*.** For the radicle assay, uniformly germinated pepper (cv., Nockwang) seeds (2–3 mm in radicle) were soaked in the bacterial suspensions for 3 h and blotted on sterile filter papers. Ten seeds per bacterial strain were placed at 28°C in the margins of 5-day-old cultures of a virulent isolate S197 of *P. capsici* on water agar (WA) containing 0.02% glucose. These treated plates were incubated at 28°C under 16 h fluorescent light/day. Seeds treated with 10 mM MgSO<sub>4</sub> solution were used as a control. Incidence (%) of infected radicles with brownish discoloration was determined 23 days after placing the seeds on WA, when almost all the

control seeds were infected. The selected strains from the initial radicle assays were tested twice again with three replicates of 10 seeds each.

For the seedling assay, germinated pepper ('Nockwang') seeds were planted in 128-cell ( $3 \times 3 \times 5$  cm) plug trays filled with potting mix (Sang et al., 2008) that included the bacterial suspensions. The bacterial strains were incorporated at  $10^8$  cells/g dry weight of potting mix. The 10 mM  $MgSO_4$  solution was used as an untreated control. The trays were placed at room temperature in a growth room under 16 h fluorescent light/day. After 2 weeks, pepper seedlings were inoculated with *P. capsici* zoospore suspension ( $2 \times 10^3$  zoospores/g dry wt. potting mix) at 1 cm below the soil-line near the seedlings. The pathogen inoculum was prepared using the procedure described by Kim et al. (1997). Disease incidence (%) was evaluated 4 days after inoculation. The selected strains from the radicle assays were tested twice with three replicates of eight seedlings each.

***In planta* trials against *P. capsici*.** Eleven bacterial strains, which were selected from the radicle and seedling assays, were tested in 5-week-old pepper ('Nockwang') plants in a growth room. Pepper seedlings, bacterial suspensions, and pathogen inocula prepared as described above were used in the *in planta* trial. Three-week-old seedlings grown in the potting mix were transplanted into steam-sterilized soil in 10 cm-diameter pots with saucers. One week later, the pots were drenched with 25 ml of each bacterial suspension. Additionally, a week later, these plants were inoculated with *P. capsici* zoospores (25 zoospores/g dry wt. soil) as follows: zoospores were injected into four holes (1 cm diameter  $\times$  1 cm deep) around each plant. These inoculated plants were continuously watered for 4–5 days through the saucers to prevent drying of the soil, and then watered as needed. Inoculated and uninoculated plants drenched with 10 mM  $MgSO_4$  solution were used as positive and negative controls, respectively. Disease severity, on a scale of 0 (symptomless) to 5 (plants dead) as described by Kim et al. (2012), was evaluated 17 and 18 days after inoculation in repeated experiments. The experiment was performed twice in a completely random design with 15 plants each.

**Field tests for Phytophthora blight and pepper fruit yield.** Field tests with the antagonistic bacterial strains (YJR27, YJR92, YJR102, and YJR107) selected from *in planta* trials were conducted at the Deokso Experiment Farm of Korea University, Namyangju, Korea from 2007 to 2009. Tests were established in beds with 7.5 m-long plots for the 2007 test and 12.5 m-long plots for the 2008 and 2009 tests, and arranged in a randomized complete block with three replicates per treatment for the 2007 test and four replicates per treatment for the 2008 and 2009 tests. Raised

beds (20 cm high  $\times$  50 cm wide), spaced 85 cm apart (center to center), were constructed and covered with black plastic mulch. Pepper plants ('Nockwang') were grown for 10 weeks in 2007, 13 weeks in 2008 and 11 weeks in 2009 in pots containing potting mix (Sang et al., 2008). The plant roots were dipped in one of the following treatments: each of the four bacterial suspensions, 10-mM  $MgSO_4$  solution (untreated control), or metalaxyl (a.i. 7.5%, Ridomil MG<sup>®</sup>, Dongbu Hannong Chemicals, Seoul, Korea) (fungicide control). These treated plants were transplanted into the beds in rows (30 cm between plants) on 1 June 2007, 16 June 2008 and 14 May 2009. Next, 10 ml of *P. capsici* ( $10^4$  zoospores/ml) was inoculated into the soil around each plant on 13 August 2007, 11 August 2008 and 22 July 2009. Disease incidence (%) and severity, as described above, were regularly evaluated after the first appearance of symptoms on the inoculated plants.

To assess root infection by *P. capsici*, three plant roots per plot were collected on 17 September 2007, 10 September 2008, and 2 September 2009. The soil attached to the collected roots was removed manually and the roots were cut into 1 cm-long fragments. The 100 root fragments (three sub-replicates per root) were placed on pimaricin-ampicillin-rifampicin-pentachloronitrobenzene-hymexazol (PARPH) medium (Solel and Pinkas, 1984) selective for *Phytophthora* spp. and incubated at 28°C. The numbers of root fragments infected by *P. capsici* were evaluated 3–4 days after placing on the medium. The experiment was conducted with three replicates of three plant roots each in 2007 and four replicates of three plant roots each in 2008 and 2009. The same root samples collected for root infection assessment were used to evaluate total populations of bacteria and fungi. The rhizosphere soil (10 g) of each plant (three sub-replicates per plant) were placed in 100 ml sterile water and shaken at 160 rpm at 28°C for 30 min. The suspension was spread and cultured at 28°C for 48 h on TSA containing 50  $\mu$ g cycloheximide/ml for total bacteria and Ohio Agricultural Experiment Station (OAES) medium (Williams and Schmitthenner, 1960) for total fungi. These experiments were conducted with the same replicates used in the root infection assessment. Bacterial and fungal colonies were counted 2 and 3 days after incubation, respectively, and assessed based on the dry weight of soil.

Pepper fruit production was evaluated to assess the plant growth-promoting effect by the selected bacteria in the 2007 and 2008 tests. Numbers and fresh weight (kg) of marketable pepper fruits (> 8 cm long), both red (ripe) and green (unripe), were counted four times after harvesting, on August 6 and 13, and September 8 and 22 in 2007, and twice on August 12 and 21 in 2008. These experiments were conducted with three replicates of 25 plants each in the 2007 test and four replicates of 40 plants each in the

2008 test.

**Plastic house tests for anthracnose and pepper fruit yield.** Anthracnose and fruit yield affected by the same selected bacterial strains were evaluated in a commercial, plastic (polyethylene; PE)-covered house (18 m wide  $\times$  45 m long) at the same University Farm in 2008 and 2009. Tests were arranged in beds with 3.6 and 4.5 m-long plots (two beds per treatment) and designed in a randomized complete block with five and four replicates per treatment in the 2008 and 2009 tests, respectively. Raised beds (20 cm high  $\times$  80 cm wide), spaced 1 m apart (center to center), were covered with black plastic mulch. Pepper plants (cv., Buja) were grown for 10 weeks in 2008 and 11 weeks in 2009 in 10 cm-diameter pots containing potting mix (Sang et al., 2008). These plants in pots were drenched once with 100 ml of each bacterial strain suspension or 10 mM MgSO<sub>4</sub> solution (untreated control) per pot on 19 April 2008 and 18 April 2009. After 2 (2009) or 3 (2008) days, plants were transplanted into the beds in rows (45 cm between plants). For pepper yield assessment, the numbers and fresh weights of marketable pepper fruits (> 8 cm long) were determined. Numbers of green (unripe) and red (ripe) fruits were counted twice, on August 2 and 25 in 2008 and on July 29 and August 19 in 2009, 2–5 days before harvesting the pepper fruits. The pepper fruit weights (red fruits only) were determined twice after harvesting, on August 4 and 27 in 2008 and on August 3 and 21 in 2009 for marketing purposes. To assess the effects of the bacterial strains against anthracnose by *C. acutatum* on pepper fruit, naturally infected green and red fruits with distinct symptoms were simultaneously evaluated at the time of fruit number evaluation. Disease incidence (%) on either the green or red fruit was determined as follows: number of infected green or red fruits divided by the total number of green or red fruits  $\times$  100. Numbers and fresh weights (kg) of pepper fruits, and anthracnose incidence were determined with five replicates of 15 plants each in the 2008 test and four replicates of 20 plants each in the 2009 test.

#### Identification of selected antagonistic bacterial strains.

Total cellular FAME analysis of the selected antagonistic strains (YJR27, YJR92, YJR102, and YJR107) was conducted by gas chromatography using the Microbial Identification System (MIDI; Newark, DE, USA) according to the manufacturer's instructions. For 16S rRNA gene sequence analysis, the genomic DNA of the strains was isolated using the i-genomic BYF DNA Extraction Mini Kit (iNtRON Biotechnology, Seongnam, Korea), and the amplicon of the 16S rRNA gene was obtained by polymerase chain reaction (PCR) using the universal primers fD1 and rP2 (Kim et al., 2012). 16S rRNA gene sequence analysis was conducted

using the BLAST network service at the National Center for Biotechnology Information (NCBI) of the U.S. National Library of Medicine (Bethesda, MD, USA). Phylogenetic trees were constructed using the neighbor-joining method and bootstrap analysis using Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.05 (The Biodesign Institute, Tempe, AZ, USA) (Saitou and Nei, 1987).

**Statistical analysis.** Statistical analysis of data was conducted using the Statistical Analysis Systems (SAS Institute, Cary, NC, USA). Data from repeated experiments in each test were pooled after confirming the homogeneity of variances with Levene's test (1960) and were further statistically analyzed. Percent data of disease incidence were statistically analyzed after arcsine square-root transformation; however, untransformed data are presented. The numbers of total bacteria and fungi in rhizosphere soils were analyzed after log transformation. For the analysis of ordinal data such as disease severity, nonparametric analysis was used based on the ranks of the data, but untransformed data are presented. Analysis of variance was determined using the general linear model procedures, and means were separated with the least significant difference (LSD) test at  $P < 0.05$ .

## Results

**Isolation of bacterial strains.** A total of 239 bacterial strains were isolated from the surface or interior of the roots and from the rhizosphere soil of cucumber, pepper, and tomato plants grown in the fields in Andong and Youngjongdo, Korea in 2004 and 2006 (Table 1).

**Selection of antagonistic bacterial strains against *P. capsici* using a sequential screening procedure.** The 239 bacterial strains showed the various levels of protection of pepper radicles against *P. capsici*. In our previous study (Sang et al., 2008), we selected strains with at least 30% radicle protection to have a better possibility of obtaining effective antagonistic strains. In this regard, 11 bacterial

**Table 1.** Sampling locations, sources and numbers of distinct bacterial strains isolated from host plants in the fields in Andong and Youngjongdo, Korea

Location	Year	Source	Host	Strain numbers isolated
Andong	2006	RI, RS, S <sup>a</sup>	Cucumber	61
		RI, RS, S	Tomato	41
Youngjongdo	2004	RI, RS	Pepper	137
Total				239

<sup>a</sup>Bacterial strains were isolated from the plant root interior (RI), root surface (RS), or rhizosphere soil (S) of the host plants.

**Table 2.** Disease incidence and severity caused by *Phytophthora capsici* on radicles, seedlings, and plants of pepper (cv., Nockwang) treated with potentially antagonistic bacterial strains

Treatment	Radicle assay <sup>a</sup> (disease incidence)	Seedling assay <sup>b</sup> (disease incidence)	<i>In planta</i> trial <sup>c</sup> (disease severity)
Untreated	96.67 ± 2.11 a <sup>d</sup>	87.50 ± 4.56 a	3.20 ± 0.20 a
YJR03	65.83 ± 2.71 b	58.93 ± 3.39 bc	2.30 ± 0.19 b
YJR07	68.33 ± 3.07 b	50.89 ± 5.25 bc	2.60 ± 0.20 ab
YJR13	54.17 ± 3.27 c-e	43.06 ± 6.43 bc	2.50 ± 0.24 b
YJR27	50.00 ± 5.77 e	40.18 ± 5.70 c	0.93 ± 0.20 cd
YJR59	65.00 ± 2.24 bc	51.19 ± 7.31 bc	2.47 ± 0.18 b
YJR89	66.67 ± 2.11 b	61.01 ± 7.67 b	2.63 ± 0.22 ab
YJR92	53.33 ± 4.22 de	53.87 ± 5.44 bc	1.00 ± 0.15 cd
YJR96	63.33 ± 2.11 b-d	41.96 ± 5.92 bc	2.43 ± 0.22 b
YJR100	63.33 ± 3.33 b-d	52.68 ± 10.74 bc	2.37 ± 0.19 b
YJR102	51.67 ± 6.54 e	58.63 ± 6.77 bc	0.70 ± 0.16 d
YJR107	66.67 ± 2.11 b	50.00 ± 8.54 bc	1.30 ± 0.23 c

<sup>a</sup>In the radicle assay, germinated seeds treated with 10-mM MgSO<sub>4</sub> solution (untreated control) or bacterial strains were placed in the margins of *P. capsici* cultures on water agar (WA) containing 0.02% glucose. Disease incidence (%) of infected radicles was determined 2–3 days after placing the seeds on WA. Values are means of six replicates with 10 seeds each from two experiments.

<sup>b</sup>Two-week-old pepper seedlings treated with the bacterial strains were inoculated with  $2 \times 10^3$  zoospores of *P. capsici* per g dry wt. potting mix. Disease incidence (%) of the seedlings was determined 4 days after inoculation. Values are means of six replicates of eight seedlings each from two experiments.

<sup>c</sup>Five-week-old pepper plants were inoculated with 25 zoospores per g dry wt. soil. Disease severity was evaluated on a scale of 0 (symptomless) to 5 (plants dead) 17 and 18 days after inoculation in two experiments. Values are means of 30 replicates from two experiments.

<sup>d</sup>Means ± standard errors within a column followed by the same letter are not significantly different according to the LSD test at  $P < 0.05$ . Arcsine square root-transformed data for disease incidence (%) on radicle and seedling assays, and the nonparametric rank test for disease severity in *in planta* trial were conducted for statistic analysis; however, untransformed data are presented.

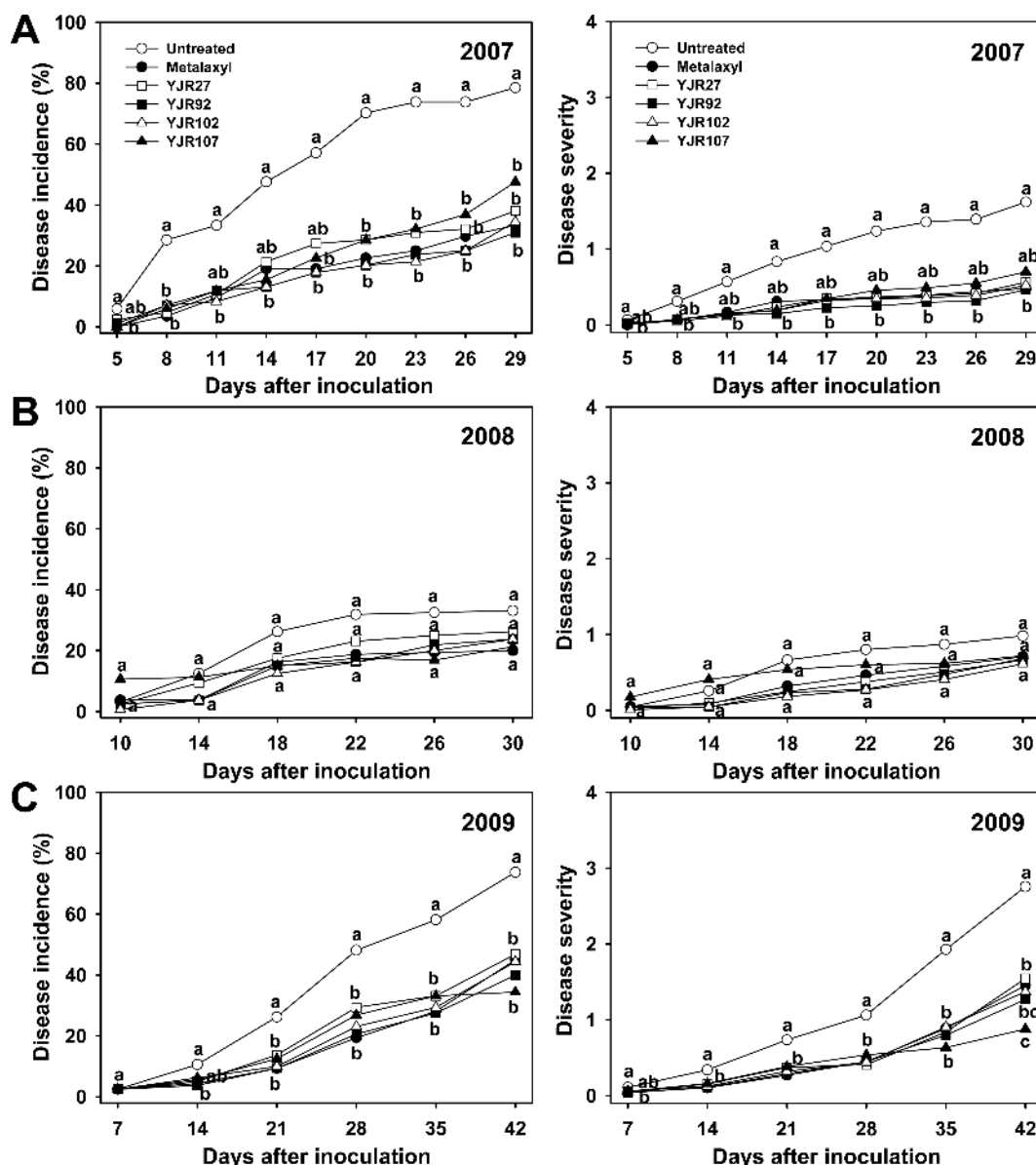
strains (Table 2) among 239 strains tested were selected as having ≤ 70% radicle infection (≥ 30% disease protection) by *P. capsici*. Among the 11 strains, four strains (YJR13, YJR27, YJR92, and YJR102) resulted in 50–60% radical infection (40–50% disease protection), and 7 strains (YJR03, YJR07, YJR59, YJR89, YJR96, YJR100, and YJR107) showed 60–70% radicle infection (30–40% disease protection) (Table 2). However, the other 228 strains showed little or no radicle protection against pathogen infection.

The 11 strains selected in this radicle assay, which were obtained from the root surfaces of pepper plants, were tested in the seedling assay. In the seedling assay, all these strains significantly ( $P < 0.05$ ) reduced disease incidence by *P. capsici*, compared with MgSO<sub>4</sub> treatment (untreated control) (Table 2). These 11 strains were then examined in

the *in planta* trial, in which nine strains, with exception of strains YJR07 and YJR89, significantly ( $P < 0.05$ ) suppressed disease severity compared with untreated controls. Consequently, four strains (YJR27, YJR92, YJR102, and YJR107) were selected as antagonistic strains for further field tests (Table 2).

**Effect of rhizobacteria on *Phytophthora* blight occurrence and pepper fruit yield in the field plants with artificial inoculum.** *Phytophthora* blight of pepper occurred severely in 2007 and 2009, and moderately in 2008, in the field plants that were artificially inoculated with *P. capsici* (Fig. 1). The final disease incidence and severity ranges were 31–77% and 0.5–1.6 in 2007, 20–33% and 0.6–1.0 in 2008, and 34–74% and 0.9–2.8 in 2009, respectively. In the 2007 test, all tested strains, with exception of strain YJR107 for disease severity, significantly ( $P < 0.05$ ) reduced disease incidence and severity, compared with MgSO<sub>4</sub> treatment (untreated control) across evaluation dates after inoculation (Fig. 1A). Strains YJR27, YJR92, and YJR102 protected pepper plants against pathogen infection at similar levels as observed in metalaxyl treatment (fungicide control). The MgSO<sub>4</sub>-treated plants produced the highest disease (disease incidence = 77% and disease severity = 1.6 at final evaluation) among all the treatments (Fig. 1A). However, in the 2008 test, moderate disease levels were observed, and none of the treatments were significantly different from untreated controls across disease evaluation dates (Fig. 1B). In the 2009 test, similar results were obtained as observed in the 2007 test (Fig. 1C). In the 2009 test, all tested strains significantly ( $P < 0.05$ ) reduced disease incidence and severity compared with untreated controls across disease evaluation dates, and showed similar or better levels in disease suppression than in metalaxyl treatment (Fig. 1C).

As a parameter of disease infection by *P. capsici*, root infection rates were assessed in field tests. In the 2007 test, all the tested strains significantly ( $P < 0.05$ ) reduced root infection in the range of 56–64%, compared with MgSO<sub>4</sub>-treated plants (untreated control) (Table 3). The results of root infection in the 2009 test were similar to those of the 2007 test; all tested strains reduced 43–62% of root infection, compared with untreated control. However, in the 2008 test, the root infection rates in all the bacterial and metalaxyl (fungicide control) treatments did not differ from those in the untreated controls. The root infection rates of MgSO<sub>4</sub>-treated controls in the 2008 test were lower than those in the 2007 and 2009 tests (Table 3). When total numbers of bacteria and fungi in the rhizosphere soils were compared, the populations of total bacteria per gram of dry soil ranged from 6.29–6.43 (log value) in 2007, 7.67–7.83 in 2008, and 7.31–7.99 in 2009 (Table 4). Likewise, popu-



**Fig. 1.** Incidence and severity of *Phytophthora* blight of pepper (cv., Nockwang) plants, in which roots were dip-treated with antagonistic bacterial strains (YJR27, YJR92, YJR102, and YJR107), metalaxyl (fungicide control), or 10-mM  $MgSO_4$  solution (untreated control) in the field at the Deokso Experiment Farm of Korea University, Namyangju, Korea in (A) 2007, (B) 2008, and (C) 2009. Means indicated by the same letter are not significantly different according to the LSD test at  $P < 0.05$ . Arcsine square root-transformed data for disease incidence (%) and the nonparametric analysis based on ranks of the data for disease severity on a scale of 0 (symptomless) to 5 (plants dead) were conducted for statistic analysis; however, untransformed data are presented.

lations of total fungi ranged from 3.11–3.26 (log value) in 2007, 4.10–4.23 in 2008, and 4.00–4.71 in 2009. Populations of total bacteria and fungi in bacteria-treated pepper rhizospheres were not significantly ( $P > 0.05$ ) different from those in  $MgSO_4$ - and metalaxyl-treated plants in test years (Table 4).

The numbers and fresh weights of pepper fruits were determined in the fields over 2 years (Tables 5 and 6). In the 2007 test, none of the tested strains yielded significantly ( $P$

$> 0.05$ ) different results compared with  $MgSO_4$  treatment (untreated control) in terms of fruit numbers at the first and second harvests (pre-inoculation) (Table 5). However, strains YJR27, YJR92, and YJR102 increased the fruit numbers at the third and fourth harvest (post-inoculation), compared with those of  $MgSO_4$ -treated controls. Metalaxyl treatment increased the numbers at the post-inoculated harvests. Similar results were obtained with regard to fruit weights. Strains YJR27, YJR92, and YJR102 as well as metalaxyl

**Table 3.** Percent pepper (cv., Nockwang) roots infected by *Phytophthora capsici*, in which the roots were dip-treated with antagonistic bacterial strains just prior to transplanting in the fields following artificial inoculation with the pathogen from 2007 to 2009

Treatment <sup>a</sup>	Infected root (%) <sup>b</sup>		
	2007	2008	2009
Untreated	78.17 ± 3.14 a <sup>c</sup>	47.33 ± 1.01 a	68.31 ± 4.70 a
Metalaxyl	33.13 ± 3.94 b	27.50 ± 10.36 a	— <sup>d</sup>
YJR27	31.57 ± 0.95 b	36.75 ± 5.10 a	38.93 ± 4.10 b
YJR92	34.07 ± 3.73 b	36.17 ± 5.22 a	27.10 ± 3.81 b
YJR102	29.53 ± 2.70 b	34.50 ± 7.82 a	27.05 ± 3.15 b
YJR107	28.00 ± 5.05 b	29.67 ± 7.60 a	25.67 ± 5.97 b

<sup>a</sup> Plant roots were dip-treated with bacterial strains, 10-mM MgSO<sub>4</sub> solution (untreated control), or metalaxyl (fungicide control) on 1 June 2007, 16 June 2008, and 14 May 2009. Plant roots were collected on 17 September 2007, 10 September 2008, and 2 September 2009.

<sup>b</sup> The percentage of infected plant roots was determined on PARPH medium selective for *Phytophthora* spp.

<sup>c</sup> Means ± standard errors within a column followed by the same letter are not significantly different according to the LSD test at  $P < 0.05$ . Arcsine square root-transformed data were used for statistical analysis; however, untransformed data are presented. These experiments were conducted with three replicates of three plants each in 2007 and four replicates of three plants each in 2008 and 2009.

<sup>d</sup> Not determined.

increased fruit weights at the fourth harvest, compared with those of the untreated control. However, strain YJR107 reduced the numbers and weights of the pepper fruit at the first and second harvests (Table 5). In the 2008 test, the fruit numbers and weights of pepper plants treated with the bacterial strains and metalaxyl did not differ from MgSO<sub>4</sub>-treated controls (Table 6).

#### Effect of rhizobacteria on anthracnose occurrence and pepper fruit yield in the plastic house plants with natural inoculum.

In the 2008 test, strains YJR27, YJR102, and

YJR107, but not strain YJR92, significantly ( $P < 0.05$ ) reduced anthracnose incidence (disease reduction rates = 57.4–73.0%) on red fruits at the second harvest, compared with MgSO<sub>4</sub> treatment (untreated control) (Fig. 2A). However, all the tested strains failed to reduce disease incidence on green fruits at the first and second harvests, as well as on red fruits at the first harvest (Fig. 2A). In the 2009 test, strain YJR92 alone significantly ( $P < 0.05$ ) reduced the disease incidence (disease reduction rate = 53.7%) on red fruit at the second harvest, compared with the untreated control (Fig. 2B). However, all the tested strains failed to reduce the disease incidence on green fruit at the first and second harvests, and on red fruit at the first harvest (Fig. 2B).

In the case of pepper fruit yield assessment, fruit numbers and fresh weights were affected by the bacterial strains in 2008 and 2009 (Table 7). In the 2008 test, strain YJR102 alone significantly ( $P < 0.05$ ) increased the numbers of red fruit at the second harvest, compared with MgSO<sub>4</sub> treatment. In terms of fresh weight of red fruit, strains YJR27 and YJR92 significantly ( $P < 0.05$ ) increased the fruit weight at the first harvest. However, all strains failed to increase the numbers of green fruit at both the harvests and of red fruit at the first harvest, as well as the red fruit weight at second harvest. Similarly, as observed in the 2008 test, in the 2009 test, all the tested strains failed to affect the numbers of green and red fruits; however, three strains (YJR27, YJR92, and YJR102) significantly ( $P < 0.05$ ) affected red fruit weights at the first harvest, but not at the second harvest (Table 7).

#### Identification of selected antagonistic bacterial strains.

The four selected strains (YJR27, YJR92, YJR102, and YJR107) were identified using FAME and 16S rRNA gene sequence analyses. From the FAME profiles, major cellular

**Table 4.** Total bacteria and fungi in the rhizosphere soils of pepper (cv., Nockwang) plants, in which the roots were dip-treated with antagonistic bacterial strains just prior to transplanting in the fields following artificial inoculation with *Phytophthora capsici* from 2007 to 2009

Treatment <sup>a</sup>	Total bacteria (log CFU/g dry wt. soil) <sup>b</sup>			Total fungi (log CFU/g dry wt. soil)		
	2007	2008	2009	2007	2008	2009
Untreated	6.39 ± 0.02 a <sup>c</sup>	7.70 ± 0.11 a	7.99 ± 0.16 a	3.11 ± 0.04 a	4.15 ± 0.04 a	4.64 ± 0.06 a
Metalaxyl	6.38 ± 0.04 a	7.72 ± 0.16 a	7.77 ± 0.12 a	3.18 ± 0.04 a	4.18 ± 0.08 a	4.71 ± 0.17 a
YJR27	6.32 ± 0.16 a	7.83 ± 0.06 a	7.89 ± 0.08 a	3.22 ± 0.06 a	4.23 ± 0.05 a	4.47 ± 0.06 a
YJR92	6.37 ± 0.10 a	7.80 ± 0.01 a	7.98 ± 0.19 a	3.24 ± 0.03 a	4.22 ± 0.03 a	4.42 ± 0.15 a
YJR102	6.43 ± 0.09 a	7.67 ± 0.07 a	7.31 ± 0.50 a	3.26 ± 0.10 a	4.18 ± 0.06 a	4.00 ± 0.14 b
YJR107	6.29 ± 0.06 a	7.68 ± 0.06 a	7.61 ± 0.31 a	3.14 ± 0.05 a	4.10 ± 0.07 a	4.41 ± 0.10 a

<sup>a</sup> Plant roots were dip-treated with bacterial strains, 10-mM MgSO<sub>4</sub> solution (untreated control), or metalaxyl (fungicide control) on 1 June 2007, 16 June 2008, and 14 May 2009. Plant roots were collected on 17 September 2007, 10 September 2008 and 2 September 2009.

<sup>b</sup> Total bacteria and fungi were determined on TSA medium containing cyclohexamide and OAES medium, respectively.

<sup>c</sup> Means ± standard errors within a column followed by the same letter are not significantly different according to the LSD test at  $P < 0.05$ . These experiments were conducted with three replicates of three plants each in 2007 and four replicates of three plants each in 2008 and 2009.

**Table 5.** Numbers and fresh weights of fruits from pepper (cv., Nockwang) plants, in which the roots were dip-treated with antagonistic bacterial strains just prior to transplanting in the field following artificial inoculation with *Phytophthora capsici* in 2007

Treatment <sup>a</sup>	Number of pepper fruit <sup>b</sup>				Fresh weight of pepper fruit (kg)			
	Pre-inoculation		Post-inoculation		Pre-inoculation		Post-inoculation	
	Harvest-1	Harvest-2	Harvest-3	Harvest-4	Harvest-1	Harvest-2	Harvest-3	Harvest-4
Untreated	144 ± 21 a <sup>c</sup>	56 ± 6 ab	57 ± 13 b	75 ± 8 c	1.91 ± 0.25 a	0.41 ± 0.04 ab	0.93 ± 0.27a-c	0.53 ± 0.06 b
Metalaxyl	104 ± 12 ab	86 ± 17 a	149 ± 9 a	169 ± 28 a	1.40 ± 0.19 ab	0.69 ± 0.15 a	1.28 ± 0.06 ab	1.14 ± 0.17 a
YJR27	93 ± 29 ab	55 ± 11 ab	101 ± 3 ab	164 ± 0 a	1.37 ± 0.48 ab	0.42 ± 0.09 ab	0.75 ± 0.02 bc	1.15 ± 0.12 a
YJR92	84 ± 16 ab	36 ± 12 b	98 ± 6 ab	137 ± 20 ab	1.07 ± 0.24 ab	0.23 ± 0.07 b	0.75 ± 0.05 bc	0.98 ± 0.07 a
YJR102	92 ± 20 ab	46 ± 12 b	154 ± 42 a	134 ± 2 ab	1.28 ± 0.36 ab	0.36 ± 0.11 b	1.38 ± 0.37 a	1.13 ± 0.17 a
YJR107	55 ± 14 b	30 ± 7 b	61 ± 17 b	106 ± 6 bc	0.71 ± 0.19 b	0.22 ± 0.07 b	0.48 ± 0.13 c	0.76 ± 0.11 ab

<sup>a</sup> Plant roots were treated with bacterial strains, 10-mM MgSO<sub>4</sub> solution (untreated control), or metalaxyl (fungicide control) on 1 June 2007.

<sup>b</sup> Numbers and fresh weights (kg) of marketable pepper fruits (> 8 cm long), both red (ripe) and green (unripe), were evaluated on August 6 and 13 as well as September 8 and 22 in 2007. After the second harvest, 1 × 10<sup>5</sup> zoospores of *P. capsici* were inoculated to the soil around each plant on 13 August 2007.

<sup>c</sup> Means ± standard errors with in a column followed by the same letter are not significantly different according to the LSD test at *P* < 0.05. These experiments were conducted with three replicates of 25 plants each.

**Table 6.** Numbers and fresh weights of fruits from pepper (cv., Nockwang) plants, in which the roots were dip-treated with antagonistic bacterial strains just prior to transplanting in the field following artificial inoculation with *Phytophthora capsici* in 2008

Treatment <sup>a</sup>	Numbers of pepper fruit <sup>b</sup>		Fresh weight of pepper fruit (kg)	
	Harvest-1	Harvest-2	Harvest-1	Harvest-2
Untreated	229 ± 31 a <sup>c</sup>	215 ± 24 a	3.10 ± 0.46 ab	1.74 ± 0.25 a
Metalaxyl	177 ± 29 a	201 ± 52 a	2.32 ± 0.36 ab	1.57 ± 0.39 a
YJR27	171 ± 29 a	163 ± 15 a	2.32 ± 0.47 ab	1.19 ± 0.13 a
YJR92	211 ± 27 a	180 ± 29 a	2.93 ± 0.42 ab	1.40 ± 0.23 a
YJR102	164 ± 28 a	231 ± 32 a	2.18 ± 0.41 b	1.59 ± 0.27 a
YJR107	242 ± 18 a	203 ± 22 a	3.48 ± 0.22 a	1.61 ± 0.18 a

<sup>a</sup> Plant roots were dip-treated with bacterial strains, 10-mM MgSO<sub>4</sub> solution (untreated control), or metalaxyl (fungicide control) on 16 June 2008.

<sup>b</sup> Numbers and fresh weights (kg) of marketable pepper fruits (> 8 cm long), both red (ripe) and green (unripe), were evaluated on August 12 and 21 in 2008. The 1 × 10<sup>5</sup> zoospores of *P. capsici* were inoculated to the soil around each plant on 11 August 2008.

<sup>c</sup> Means ± standard errors with in a column followed by the same letter are not significantly different according to the LSD test at *P* < 0.05. These experiments were conducted with four replicates of 40 plants each.

fatty acids of strain YJR27 were C<sub>16:0</sub> (22.9%), summed feature 3 (C<sub>16:1</sub> ω7c and/or C<sub>16:1</sub> ω6c) (20.3%), and C<sub>12:0</sub> (12.1%) and those of strain YJR92 were C<sub>16:0</sub> (30.6%), summed feature 3 (C<sub>16:1</sub> ω7c and/or C<sub>16:1</sub> ω6c) (20.2%), and summed feature 8 (C<sub>18:1</sub> ω7c) (18.5%) which identified the strain as belonging to the genus *Pseudomonas*. Strain YJR102 mainly had C<sub>18:1</sub> ω9c (34.7%), C<sub>16:0</sub> (31.2%), and summed feature 3 (C<sub>16:1</sub> ω6c and/or C<sub>16:1</sub> ω7c) (17.2%), and was identified as belonging to the genus *Tsukamurella*. On the other hand, strain YJR107 had C<sub>18:1</sub> ω7c (57.7%) and

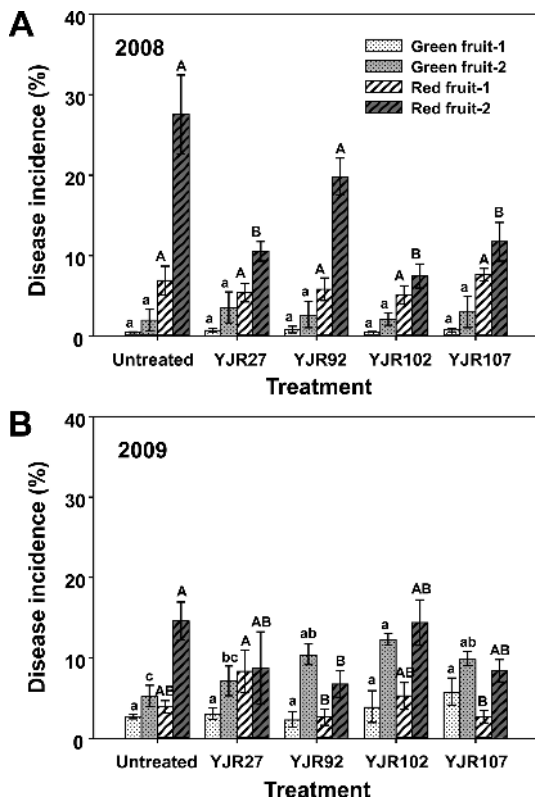
C<sub>14:0</sub> 2OH (14.9%), and was identified as belonging to the genus *Novosphingobium* or related genus. Further, 16S rRNA gene sequence analysis identified strains YJR27 as *Pseudomonas otitidis* (similarity = 99%), YJR92 as *Pseudomonas putida* (99%), YJR102 as *Tsukamurella tyrosinosolvens* (100%), and YJR107 as *Novosphingobium capsulatum* (99%). The 16S rRNA gene sequences of strains YJR27 (accession number = JX154555), YJR92 (JX154556), YJR102 (JX154557), and YJR107 (JX154554) have been deposited in the GenBank.

## Discussion

We previously described a sequential screening procedure requiring reduced time and labor, consisting of radicle and seedling assays and *in planta* trials, to select effective antagonistic bacterial strains against *P. capsici* in pepper plants (Kim et al., 2008a). In this study, we selected four strains from 239 tested strains as potential biocontrol agents against the pathogen using this screening procedure. Thereafter, we assessed the control efficacy of these selected strains against *Phytophthora* blight of pepper under field conditions with artificial inoculation from 2007 to 2009, and against anthracnose under plastic house conditions with natural inoculation in 2008 and 2009. Further, we evaluated the effect of these strains on pepper fruit yield under the same conditions. The results from this study confirmed that the developed screening procedure was reliable and rapid for selecting antagonistic bacterial strains against *P. capsici* in pepper plants. In addition, these selected strains exhibited biocontrol activities against anthracnose, and some of strains also exhibited plant growth-promotion effects on pepper fruit yield.

We selected four bacterial strains (*P. otitidis* YJR27, *P.*





**Fig. 2.** Incidence of anthracnose on green and red fruits from pepper (cv., Buja) plants after transplanting seedlings, in which the roots were dip-treated with antagonistic bacterial strains (YJR27, YJR92, YJR102, and YJR107) or 10 mM MgSO<sub>4</sub> solution (untreated control) at the plastic house at the Deokso Experiment Farm of Korea University, Namyangju, Korea in (A) 2008 and (B) 2009. These seedlings were transplanted into the beds on 22 April 2008 and 20 April 2009. Disease incidence (%) was evaluated on pepper fruit on August 2 (first evaluation) and 25 (second evaluation) in 2008 and on July 29 (first evaluation) and August 19 (second evaluation) in 2009. The bars are means of five replicates with 15 plants each in 2008 and four replicates with 20 plants each in 2009. Bar with error bars followed by the same letters (small and capital letters for green and red fruits, respectively) are not significantly different between treatments according to the LSD test at  $P < 0.05$ . Arcsine square root-transformed data were used for the statistical analysis of disease incidence (%); however, untransformed data are presented.

*putida* YJR92, *T. tyrosinosolvens* YJR102, and *N. capsulatum* YJR107) from the sequential screening procedure as potential biocontrol agents against the pepper disease. However, in selecting these strains in this study, we used a modified screening procedure (in terms of the baseline of the radicle assay and the inoculum concentration in the seedling assay) (Sang et al., 2008) from the initially developed procedure (Kim et al., 2008a) to obtain better antagonistic strains. For example, the initial screening baseline of the radicle assay was  $\leq 80\%$  radicle infection ( $\geq 20\%$  radicle protection) but the baseline was increased to  $\leq 70\%$  radicle

infection ( $\geq 30\%$  radicle protection) in this modified procedure. In addition, the inoculum concentration for the seedling assay was increased to  $2 \times 10^3$  zoospores from  $1 \times 10^3$  zoospores per gram dry weight of potting mix. From our previous study (Sang et al., 2008), we found that these severe selection pressures could significantly reduce the time and labor required to select appropriate antagonistic strains, and this was subsequently evaluated in *in planta* trials and field tests.

Further, we confirmed the merit of the modified procedure for selecting potential biocontrol strains. When comparing the initial baseline of 80% radicle infection (20% radicle protection) to the modified baseline of 70% (30% radicle protection) in the radicle assay, 88 of 231 strains (38.1%) were selected as potentially antagonistic strains using the 80% radicle infection in our initial study (Kim et al., 2008a). However, using the baseline of 70% radicle infection, 38 of 439 strains (8.4%) (Sang et al., 2008), and 11 of 239 strains (4.6%) in this study were selected as potentially antagonistic strains. Eventually, using the screening procedure, 11 of 231 strains (4.8%) (Kim et al., 2008a), 16 of 439 strains (3.6%) (Sang et al., 2008), and nine of 239 strains (3.8%) in this study were selected as antagonistic strains against *P. capsici*. In addition, these consistent results might be derived from the merit of the radicle assay as a pre-screening method. Since the radicle assay was designed to evaluate the interaction between the bacterial strain (antagonist), pepper radicle (host), and *P. capsici* (pathogen), strains from this assay have a good potential for survival and persist on the root surface or in the rhizosphere, which could play a significant role in disease suppression in the seedling assay and *in planta* trials. Recently, Barahona et al. (2011) indicated that the activity of biocontrol agents could be improved by increasing their competitive colonization ability. Therefore, the sequential screening procedure based on the pre-screening radicle assay could offer not only rapid selection but also increased colonization ability of the selected strains against *P. capsici* in pepper plants.

In the 3-year field tests, the control efficacies of the selected strains (YJR27, YJR92, YJR102, and YJR107) against Phytophthora blight of pepper were examined. In the 2007 and 2009 tests, three strains YJR27, YJR92, and YJR102 exhibited significant biocontrol abilities against the disease; however, in the 2008 test, all the strains failed to reduce it. The disease reductions in the 2007 and 2009 tests were more obvious when observed in terms of root infection rates by *P. capsici*. The tested strains greatly reduced root infection by the pathogen relative to the untreated control, and showed similar efficacies as seen in plants treated with metalaxyl in the 2007 and 2009 tests, but not in the 2008 test. Our previous studies (Kim et al., 2009,

**Table 7.** Numbers and fresh weights of green (unripe) and red (ripe) fruits of pepper (cv., Buja) plants, in which the roots were dip-treated with antagonistic bacterial strains in the plastic house in 2008 and 2009

Year, treatment <sup>a</sup>	Number of pepper fruit <sup>b</sup>				Fresh weight of red pepper fruit (kg) <sup>c</sup>	
	Green fruit		Red fruit		Harvest-1	Harvest-2
	Harvest-1	Harvest-2	Harvest-1	Harvest-2		
<b>2008</b>						
Untreated	195 ± 19 a <sup>d</sup>	245 ± 10 a	243 ± 15 a	201 ± 16 b	4.08 ± 0.20 b	3.96 ± 0.20 a
YJR27	206 ± 11 a	239 ± 24 a	213 ± 27 a	230 ± 11 ab	4.92 ± 0.30 a	3.90 ± 0.46 a
YJR92	213 ± 20 a	274 ± 17 a	237 ± 26 a	226 ± 18 ab	5.24 ± 0.27 a	3.70 ± 0.34 a
YJR102	201 ± 22 a	264 ± 25 a	221 ± 22 a	261 ± 17 a	4.62 ± 0.33 ab	4.02 ± 0.42 a
YJR107	191 ± 10 a	287 ± 24 a	239 ± 17 a	231 ± 22 ab	4.48 ± 0.17 ab	3.52 ± 0.37 a
<b>2009</b>						
Untreated	204 ± 21 a	86 ± 10 a	260 ± 20 a	160 ± 19 a	2.70 ± 0.06 b	2.10 ± 0.42 a
YJR27	214 ± 31 a	122 ± 31 a	252 ± 11 a	208 ± 44 a	4.00 ± 0.37 a	3.50 ± 0.39 a
YJR92	243 ± 63 a	125 ± 23 a	250 ± 15 a	264 ± 61 a	4.03 ± 0.53 a	3.48 ± 0.66 a
YJR102	225 ± 28 a	149 ± 30 a	261 ± 12 a	219 ± 33 a	4.38 ± 0.45 a	3.38 ± 0.09 a
YJR107	207 ± 44 a	119 ± 18 a	220 ± 33 a	188 ± 49 a	3.90 ± 0.34 ab	2.90 ± 0.50 a

<sup>a</sup> Plant seedlings were treated with bacterial strains, 10 mM MgSO<sub>4</sub> solution (untreated control), or metalaxyl (fungicide control) on 19 April 2008 and 18 April 2009. These seedlings were transplanted into the beds on 22 April 2008 and 20 April 2009.

<sup>b</sup> The numbers of marketable green and red fruit (> 8 cm long) were evaluated on August 2 and 25 in 2008 and on July 29 and August 19 in 2009.

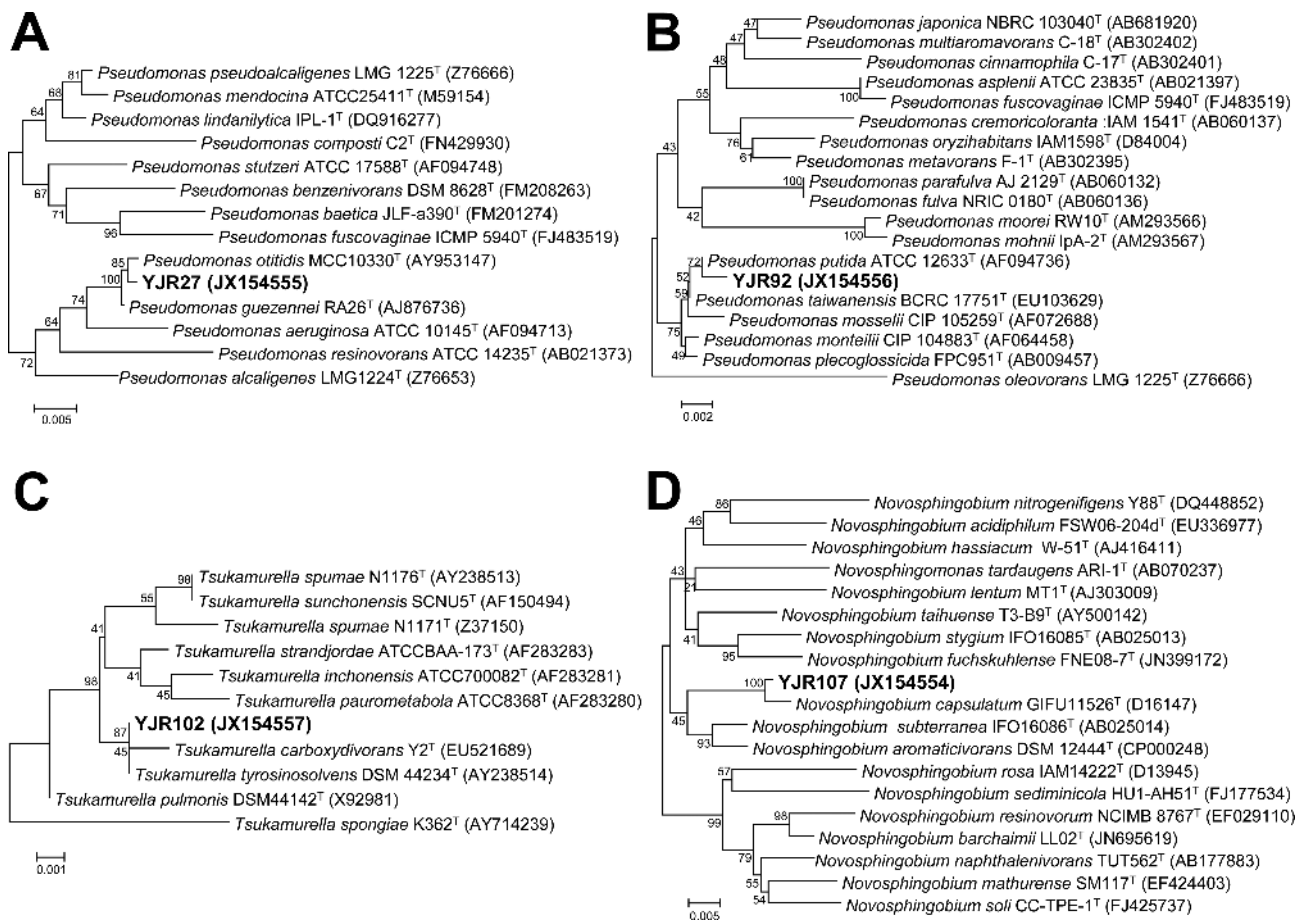
<sup>c</sup> Fresh weights (kg) of marketable red pepper fruit (> 8 cm long) were determined on August 4 and 27 in 2008 and August 3 and 21 in 2009.

<sup>d</sup> Values are means of five replicates of 15 plants each in 2008 and four replicates of 20 plants each in 2009. Mean ± standard errors followed by the same letters are not significantly different between treatments according to the LSD test at  $P < 0.05$ .

2012; Sang and Kim, 2012a) demonstrated that root protection through colonization by biocontrol strains against *P. capsici* could play an important role in disease suppression. However, in the 2008 test, the disease occurrence and root infection rates were not significantly different between the strain-treated plants and untreated or metalaxyl-treated control plants. The lack of significant difference in disease suppression might be due to low disease pressure in 2008, compared with severe disease pressure in the 2007 and 2009 tests; this could be affected by various environmental factors. Recently, Seo et al. (2011) reported that the occurrence of this pepper disease in the fields in Korea was lower in 2008 than in 2007. In addition, it was shown that the tested strains did not negatively affect the microbial population in the pepper rhizosphere when the influence of the strains on total bacterial and fungal populations was examined. It is desirable for the introduced biocontrol strains not to alter the microbial community or diversity in the environment. However, since only culturable bacteria and fungi on media were assessed in this study, further examination of the effect of these strains on the root and soil microbial community and diversity, including non-culturable microbes, is needed as studied by Sang and Kim (2012b).

In the 2-year plastic house tests, the tested same strains reduced anthracnose incidence by *C. acutatum* (Sang et al., 2011), possibly through ISR as observed in our previous

works (Sang and Kim, 2011; Sang et al., 2011), at least one of test years but their biocontrol activities were variable. These variable results might be due to the limitations of the required populations of the treated strains for inducing effective ISR against anthracnose in pepper fruit. Recently, Nijhuis et al. (2010) suggested that a sufficient population of *Lysobacter enzymogenes* 3.IT8 on cucumber roots was required for the biocontrol of *Pythium* root rot. Consistency and improvement in the microbial biocontrol activity can be gained by the use of certain additional compounds (Shaukat and Siddiqui, 2003). Kim et al. (2008b) reported that the biocontrol activity of antagonistic *Chryseobacterium* sp. KJ1R5 against *P. capsici* could be enhanced by the addition of certain carbon sources. In another study, Postma et al. (2009) found that higher numbers of *L. enzymogenes* 3.IT8 were obtained by adding chitosan, resulting in effective control of *Pythium aphanidermatum*. Thus, the biocontrol activities of bacterial strains against target diseases could be expressed consistently and effectively through the enhancement or maintenance of the populations of the antagonistic strains. Accordingly, the tested strains in this study may need to preserve certain population levels in the pepper rhizosphere or root to consistently elicit ISR against *C. acutatum* infection. Therefore, to achieve consistent biocontrol efficacy against anthracnose and Phytophthora blight of pepper in fields or plastic houses, which exhibit dynamic environmental changes, careful evaluation of the temporal



**Fig. 3.** Neighbor-joining phylogenetic trees based on 16S rRNA gene sequence analysis for strains (A) YJR27, (B) YJR92, (C) YJR102, and (D) YJR107 with the type strains of closely related species at the similarity levels of  $\geq 97$ , 98, 97, and 95%, respectively. Numbers at nodes indicate bootstrap values, expressed as percentages of 1000 replications. The type strain of the species is indicated as “T”; accession numbers at the GenBank are shown in parentheses.

population changes of the treated strains in pepper rhizosphere or root is needed.

In this study, three of four selected strains (YJR27, YJR92, and YJR102) increased pepper fruit numbers after inoculation in the field tests, and these strains increased red fruit weights at the first harvest in the plastic house tests. The increased fruit yield might be dependent on disease reduction by the antagonistic strains, because yield reduction was not observed before inoculation. Moreover, the plastic house tests revealed that the strains exhibited plant growth-promoting ability in terms of pepper fruit yield. It is known that plant growth promotion can be achieved directly by enhancing the utilization of nutrients and minerals, or by regulating plant hormones including indole-3-acetic acid (IAA), cytokinins, and ethylene or indirectly by disease suppression via biocontrol agents (Gray and Smith, 2005; Mia et al., 2010; Shanmugam et al., 2011; Zheng et al., 2011). Thus, these results show that the strains YJR27, YJR92, and YJR102 have the ability to improve plant growth

and health by protecting against the tested plant diseases in this study.

When these selected strains were identified using FAME and 16S rRNA gene sequence analyses, strain YJR27 was determined as *P. otitidis*, strain YJR92 as *P. putida*, strain YJR102 as *T. tyrosinosolvans* and strain YJR107 as *N. capsulatum*. It is well known that many strains belonging to the genus *Pseudomonas* have biological traits such as antibiotic production, competitive colonization, ISR, and plant growth promotion (Weller, 2007). Within the genus *Pseudomonas*, *P. putida* has been reported as an effective biocontrol bacterial species against various plant diseases including Rhizoctonia and Pythium root rot of wheat (Mavrodi et al., 2012). Likewise, *P. otitidis* has been found in various environments including water waste, and can produce *N*-acylhomoserine lactones (AHLs), IAA, and bacteriocin (Mehri et al., 2011). However, *T. tyrosinosolvans*, which belongs to Actinomycetes, has not previously been reported as an antagonistic bacterial species against plant

diseases; however, a strain of species belonging to the genus *Tsukamurella* has been isolated from the gut of Japanese honeybee (*Apis cerana japonica*) (Yoshiyama and Kumura, 2009), and from the samples of patients with cardiac pacemaker implants and chronic lung infections (Yassin et al., 1997). In the case of *N. capsulatum*, Halfeld-Vieira et al. (2008) observed that the species was capable of reducing late blight of tomato caused by *Phytophthora infestans* under field condition. The results of bacterial identification of the selected strains, therefore, indicate that the use of the sequential screening procedure has resulted in the selection of diverse bacterial resources for controlling pepper diseases and promoting plant growth.

Taken together, these results indicate that the sequential screening procedure is a rapid and reliable method to select effective antagonistic bacterial strains against *P. capsici* in pepper plants. This screening procedure allowed us to obtain four effective biocontrol strains (*P. otitidis* YJR27, *P. putida* YJR92, *T. tyrosinosolvens* YJR102, and *N. capsulatum* YJR107) against Phytophthora blight of pepper. These strains also had biocontrol activities against anthracnose, and the strains YJR27, YJR92, and YJR102 also showed plant growth-promoting activities on pepper fruit. Thus, these bacterial strains may be promising biocontrol agents against Phytophthora blight and anthracnose of pepper.

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### References

- Ahmadzadeh, M. and Tehrani, A. S. 2009. Evaluation of fluorescent pseudomonads for plant growth promotion, antifungal activity against *Rhizoctonia solani* on common bean, and biocontrol potential. *Biol. Control* 48:101–107.
- Barahona, E., Navazo, A., Martínez-Granero, F., Zea-Bonilla, T., Pérez-Jiménez, R. M., Martín, M. and Rivilla, R. 2011. *Pseudomonas fluorescens* F113 mutant with enhanced competitive colonization ability and improved biocontrol activity against fungal root pathogens. *Appl. Environ. Microbiol.* 77:5412–5419.
- Bardas, G. A., Lagopodi, A. L., Kadoglidou, K. and Tzavella-Klonari, K. 2009. Biological control of three *Colletotrichum lindemuthianum* races using *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas fluorescens* WCS365. *Biol. Control* 49:139–145.
- Chanchaichaovivat, A., Panijpan, B. and Ruenwongsa, P. 2008. Putative modes of action of *Pichia guilliermondii* strain R13 in controlling chilli anthracnose after harvest. *Biol. Control* 47:207–215.
- Chang, S. H., Kwack, M. S., Kim, Y. S., Lee, J. Y. and Kim, K. D. 2001. A rapid radicle assay for prescreening antagonistic bacteria against *Phytophthora capsici* on pepper. *Mycobiology* 29:218–223.
- Choudhary, D. K. and Johri, B. N. 2008. Interaction of *Bacillus* spp. and plants with special reference to induced systemic resistance (ISR). *Microbiol. Res.* 164:493–513.
- Cohen, Y. and Coffey, M. D. 1986. Systemic fungicides and the control of oomycetes. *Annu. Rev. Phytopathol.* 24:311–338.
- French-Monar, R. D., Jones, J. B. and Roberts, P. D. 2006. Characterization of *Phytophthora capsici* associated with roots of weeds on Florida vegetable farms. *Plant Dis.* 90:345–350.
- Fu, G., Huang, S. L., Ye, Y. F., Wu, Y. G., Cen, Z. L. and Lin, S. H. 2010. Characterization of a bacterial biocontrol strain B106 and its efficacies in controlling banana leaf spot and post-harvest anthracnose diseases. *Biol. Control* 55:1–10.
- Gray, E. J. and Smith, D. L. 2005. Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. *Soil Biol. Biochem.* 37:395–412.
- Halfeld-Vieira, B. A., Romeiro, R. S., Mounteer, A. and Mizubuti, E. S. G. 2008. Efficiency of phylloplane bacteria in controlling aerial tomato diseases under field conditions. *Summa Phytopathol.* 34:86–87.
- Hausbeck, M. K. and Lamour, K. H. 2004. *Phytophthora capsici* on vegetable crops: research progress and management challenges. *Plant Dis.* 88:1292–1303.
- Hwang, B. K. and Kim, C. H. 1995. Phytophthora blight of pepper and its control in Korea. *Plant Dis.* 79:221–227.
- Kim, K. D., Nemeč, S. and Musson, G. 1997. Control of Phytophthora root and crown rot of bell pepper with composts and soil amendments in the greenhouse. *Appl. Soil Ecol.* 5:169–179.
- Kim, H. S., Sang, M. K., Jeun, Y. C., Hwang, B. K. and Kim, K. D. 2008a. Sequential selection and efficacy of antagonistic rhizobacteria for controlling Phytophthora blight of pepper. *Crop Prot.* 27:436–443.
- Kim, H.-S., Sang, M. K., Jung, H. W., Jeun, Y.-C., Myung, I.-S. and Kim, K. D. 2012. Identification and characterization of *Chryseobacterium wanjuae* strain KJ9C8 as a biocontrol agent against Phytophthora blight of pepper. *Crop Prot.* 32:129–137.
- Kim, H.-S., Sang, M. K., Myung, I. S., Chun, S. C. and Kim, K. D. 2009. Characterization of *Bacillus luciferensis* strain KJ2C12 from pepper root, a biocontrol agent of Phytophthora blight of pepper. *Plant Pathol. J.* 25:62–69.
- Kim, Y. S., Jang, B., Chung, I.-M., Sang, M. K., Ku, H.-M. Kim, K. D. and Chun, S.-C. 2008b. Enhancement of biocontrol activity of antagonistic *Chryseobacterium* strain KJ1R5 by adding carbon sources against *Phytophthora capsici*. *Plant Pathol. J.* 24:164–170.
- Kloepper, J. W., Ryu, C.-M. and Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94:1259–1266.
- Lamour, K. H. and Hausbeck, M. K. 2000. Mefenoxam insensitivity and the sexual stage of *Phytophthora capsici* in Michigan cucurbit fields. *Phytopathology* 90:396–400.

- Lamour, K. H. and Hausbeck, M. K. 2003. Susceptibility of mefenoxam-treated cucurbits to isolates of *Phytophthora capsici* sensitive and insensitive to mefenoxam. *Plant Dis.* 87:920–922.
- Landa, B. B., Navas-Cortés, J. A. and Jiménez-Díaz, R. M. 2004. Influence of temperature on plant-rhizobacteria interaction related to biocontrol potential for suppression of Fusarium wilt of chickpea. *Plant Pathol.* 53:341–352.
- Levene, H. 1960. Contributions to Probability and Statistics: Essays in Honor of Harold Hotelling. Stanford University Press, Stanford, CA, USA.
- Maleki, M., Mokhtarnejad, L. and Mostafaei, S. 2011. Screening of rhizobacteria for biological control of cucumber root and crown rot caused by *Phytophthora drechsleri*. *Plant Pathol. J.* 27:78–84.
- Mavrodi, O. V., Walter, N., Elateek, S., Taylor, C. G. and Okubara, P. A. 2012. Suppression of Rhizoctonia and Pythium root rot of wheat by new strains of *Pseudomonas*. *Biol. Control* 62:93–102.
- Mehri, I., Turki, Y., Chair, M., Chérif, H., Hassen, A., Meyer, J.-M. and Gtari, M. 2011. Genetic and functional heterogeneities among fluorescent *Pseudomonas* isolated from environmental samples. *J. Gen. Appl. Microbiol.* 57:101–114.
- Mia, M. A. B., Shamsuddin, Z. H., Wahab, Z. and Marziah, M. 2010. Rhizobacteria as bioenhancer and biofertilizer for growth and yield of banana (*Musa* spp. cv. 'Berangan'). *Sci. Hortic.* 126:80–87.
- Neher, O. T., Johnston, M. R., Zidack, N. K. and Jacobsen, B. J. 2009. Evaluation of *Bacillus mycoides* isolate BmJ and *B. mojavensis* isolate 203-7 for the control of anthracnose of cucurbits caused by *Glomerella cingulata* var. *orbiculare*. *Biol. Control* 48:140–146.
- Nijhuis, E. H., Pastoor, R. and Postma, J. 2010. Specific detection of *Lysobacter enzymogenes* (Christensen and Cook 1978) strain 3.IT8 with TaqMan<sup>®</sup> PCR. *J. Appl. Microbiol.* 108: 1155–1166.
- Park, K. S. and Kim, C. H. 1992. Identification, distribution and etiological characteristics of anthracnose fungi of red pepper in Korea. *Korean J. Plant Pathol.* 8:61–69.
- Parra, G. and Ristaino, J. B. 2001. Resistance to mefenoxam and metalaxyl among field isolates of *Phytophthora capsici* causing Phytophthora blight of bell pepper. *Plant Dis.* 85:1069–1075.
- Perazzolli, M., Dagostin, S., Ferrari, A., Elad, Y. and Pertot, I. 2008. Induction of systemic resistance against *Plasmopara viticola* in grapevine by *Trichoderma harzianum* T39 and benzothiadiazole. *Biol. Control* 47:228–234.
- Peres, N. A., Timmer, L. W., Adaskaveg, J. E. and Correll, J. C. 2005. Lifestyles of *Colletotrichum acutatum*. *Plant Dis.* 89: 784–796.
- Postma, J., Stevens, L. H., Wieggers, G. K., Davelaar, E. and Nijhuis, E. H. 2009. Biological control of *Pythium aphanidermatum* in cucumber with a combined application of *Lysobacter enzymogenes* strain 3.IT8 and chitosan. *Biol. Control* 48:301–309.
- Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–425.
- Sang, M. K., Chun, S.-C. and Kim, K. D. 2008. Biological control of Phytophthora blight of pepper by antagonistic rhizobacteria selected from a sequential screening procedure. *Biol. Control* 46:424–433.
- Sang, M. K., Kim, J. D., Kim, B. S. and Kim, K. D. 2011. Root treatment with rhizobacteria antagonistic to Phytophthora blight affects anthracnose occurrence, ripening, and yield of pepper fruit in the plastic house and field. *Phytopathology* 101:666–678.
- Sang, M. K., Kim, J. G. and Kim, K. D. 2010. Biocontrol activity and induction of systemic resistance in pepper by compost water extracts against *Phytophthora capsici*. *Phytopathology* 100:774–783.
- Sang, M. K. and Kim, K. D. 2011. Biocontrol activity and primed systemic resistance by compost water extracts against anthracnoses of pepper and cucumber. *Phytopathology* 101:732–740.
- Sang, M. K. and Kim, K. D. 2012a. The volatile-producing *Flavobacterium johnsoniae* strain GSE09 shows biocontrol activity against *Phytophthora capsici* in pepper. *J. Appl. Microbiol.* 113:383–398.
- Sang, M. K. and Kim, K. D. 2012b. Plant growth-promoting rhizobacteria suppressive to Phytophthora blight affect microbial activities and communities in the rhizosphere of pepper (*Capsicum annuum* L.) in the field. *Appl. Soil Ecol.* 62:88–97.
- Sang, M. K., Oh, J. Y. and Kim, K. D. 2007. Root-dipping application of antagonistic rhizobacteria at transplanting for the control of Phytophthora blight of pepper under field conditions. *Plant Pathol. J.* 23:109–112.
- Seo, J. A., Yi, Y. K., Kim, B. S., Hwang, J. M. and Choi, S. W. 2011. Disease occurrence on red-pepper plants surveyed in northern Kyungbuk province, 2007–2008. *Res. Plant Dis.* 17:205–210.
- Shanmugam, V., Kanoujia, N., Singh, M., Singh, S. and Prasad, R. 2011. Biocontrol of vascular wilt and corm rot of gladiolus caused by *Fusarium oxysporum* f. sp. *gladioli* using plant growth promoting rhizobacterial mixture. *Crop Prot.* 30:807–813.
- Shaukat, S. S. and Siddiqui, I. A. 2003. The influence of mineral and carbon sources on biological control of charcoal rot fungus, *Macrophomina phaseolina* by fluorescent pseudomonads in tomato. *Lett. Appl. Microbiol.* 36:392–398.
- Solel, Z. and Pinkas, Y. 1984. A modified selective medium for detecting *Phytophthora cinnamomi* on avocado roots. *Phytopathology* 74:506–508.
- Tian, D. and Babadoost, M. 2004. Host range of *Phytophthora capsici* from pumpkin and pathogenicity of isolates. *Plant Dis.* 88:485–489.
- Weller, D. M. 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. *Phytopathology* 97:250–256.
- Williams, L. E. and Schmitthenner, A. F. 1960. Effect of growing crops and crop residues on soil fungi and seedling blights. *Phytopathology* 50:22–25.

- Yassin, A. F., Rainey, F. A., Burghardt, J., Brzezinka, H., Schmitt, S., Seifert, P., Zimmermann, O., Mauch, H., Gierth, D., Lux, I. and Schaal, K. P. 1997. *Tsukamurella tyrosinosolvans* sp. nov. *Int. J. Syst. Bacteriol.* 47:607–614.
- Yoshiyama, M. and Kimura, K. 2009. Bacteria in the gut of Japanese honeybee, *Apis cerana japonica*, and their antagonistic effect against *Paenibacillus larvae*, the causal agent of American foulbrood. *J. Invertebr. Pathol.* 102:91–96.
- Zhang, S., White, T. L., Martinez, M. C., McInroy, J. A., Kloeper, J. W. and Klassen, W. 2010. Evaluation of plant growth-promoting rhizobacteria for control of Phytophthora blight on squash under greenhouse conditions. *Biol. Control* 53:129–135.