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Biological control of Phytophthora blight of pepper by antagonistic rhizobacteria selected from a sequential screening procedure

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Abstract

This study was conducted to select antagonistic rhizobacteria against *Phytophthora capsici* using a sequential screening procedure and to evaluate control efficacy of drench or root-dip treatments with the selected strains against Phytophthora blight of pepper in the field. Out of 439 bacterial strains, 16 potentially antagonistic strains were screened through radicle and seedling assays and *in planta* trials, and five candidate strains, CCR04, CCR80, GSE09, ISE13, and ISE14, were selected for field tests. In 2005 and 2006 tests, the control efficacy of the five strains was examined against Phytophthora blight of pepper plants drenched with the bacterial suspension in artificial pathogen inoculation. Three strains, CCR04, CCR80, and ISE14, consistently reduced the disease in both tests. As another form of application, the control efficacy of root-dip treatment was examined on pepper plants just prior to transplanting in the field with natural inoculation in 2006 and 2007. In these tests, four strains, CCR04, CCR80, GSE09, and ISE14, showed consistently good control efficacy against *P. capsici*, and strains CCR80 and ISE14 increased pepper fruit yield. The strain-treated roots had less infection rates by *P. capsici* compared with control roots regardless of drench or root-dip treatments. In addition, the strains did not affect the populations of bacteria and fungi in the rhizosphere soil. Therefore, the antagonistic strains selected from the screening procedure provided significant protection against *P. capsici* through pepper root colonization. These strains could be applied by either drench or root-dip treatment as alternatives to agricultural chemicals to control Phytophthora blight of pepper.

Keywords: Antagonistic bacteria; Biological control; Pepper; *Phytophthora capsici*

1. Introduction

Pepper (*Capsicum annum* L.) is one of the most important market vegetables grown worldwide, but the yield and quality of marketable peppers are frequently limited by Phytophthora blight caused by the oomycete pathogen *Phytophthora capsici* (Hwang and Kim, 1995; Ristaino and Johnston, 1999; Hausbeck and Lamour, 2004). The incidence of this disease has continued to increase worldwide since the pathogen can infect roots, crowns and even foliar parts of pepper plants through splashing rains or overhead irrigation waters (Ristaino and Johnston, 1999; Hausbeck and Lamour, 2004). Control of this disease has usually depended on chemical and cultural measures such as use of phenylamide fungicides, metalaxyl or fosetyl-Al as well as crop rotation, soil amendments, use of protective mulches and water management (Matheron and Porchas, 2000; Hausbeck and Lamour, 2004). However, these controls sometimes do not produce desirable disease control efficacy due to the persistence of oospore propagules of *P. capsici* and the occurrence of fungicide resistance (Lamour and Hausbeck, 2000; Matheron and Porchas, 2000; Parra and Ristaino, 2001). In addition, application of fungicides and fumigants are costly and can be biohazardous to the environment and harmful to crops (Cohen and Coffey, 1986).

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As an alternative for these control measures, biological control with many different types of microorganisms has been regarded as an attractive, environmental-sound alternative for plant disease control (Berg et al., 2001; Bloemberg and Lugtenberg, 2001; Heungens and Parke, 2001; Whipps, 2001; Georgakopoulos et al., 2002; Shen et al., 2007). In spite of the development of many biocontrol agents, it is difficult to maintain consistent control of plant diseases under various field conditions. This inconsistency may be due to either inappropriate screening or application of microorganisms most suitable for disease control in the diverse field conditions. Microbial control efficacy is frequently dependent on various abiotic and biotic factors such as soil microbial biomass and activity, soil moisture, temperature, and nutrients in host and pathogen interactions (Hase et al., 1999; Shaukat and Siddiqui, 2003; Landa et al., 2004; Bae and Knudsen, 2005). Therefore, microorganisms as biocontrol agents must be adapted to a certain environment with various biotic and abiotic factors. Consequently, practical procedures for their applications are needed to achieve effective biocontrol (Chatterton et al., 2004). For this purpose, application methods such as seed treatments, transplant amendments, spraying, or combinations of biocontrol agents have been researched to introduce the agents screened through laboratory screening into field conditions (Mao et al., 1998; Siddiqui and Shaukat, 2003; Jiang et al., 2006; Kokalis-Burelle et al., 2006; Ryu et al., 2006).

Recently, we developed a sequential screening procedure for selecting effective antagonistic bacteria against Phytophthora blight of pepper (Kim et al., 2008). This procedure has proven to be appropriate and effective in screening biocontrol agents against the disease caused by *P. capsici*. Therefore, the objectives of this study were to select antagonistic rhizobacteria against *P. capsici* using the sequential screening procedure and to evaluate the control efficacy of drench and root-dip treatments of the selected rhizobacteria against Phytophthora blight of pepper in the field with artificial and natural inoculations, respectively.

2. Materials and methods

2.1. Isolation and preparation of bacterial strains

Bacterial strains were isolated from rhizosphere soils, root surfaces, and root interiors of cucumber, pepper, and tomato plants grown in fields in 12 locations in Korea from 2001 to 2003 (Fig. 1 and Table 1). Samples of plant roots with soils were collected in polyethylene bags, stored in an ice chest, and used within several days after collection.

To obtain bacterial strains from rhizosphere soils, 10 g of soil were placed in 100 ml of sterile water; 10 g of roots with soil removed were used to isolate bacterial strains from root surfaces. To isolate strains from root interiors, 10 g of roots were surface-sterilized with 1% sodium hypochlorite for 90 s, rinsed several times in sterile distilled water, and completely macerated with a sterile homogenizer. These macerated roots were placed in 100 ml of sterile water and shaken at 160 rpm at 28 °C for 30 min. The suspension was cultured on tryptic soy agar (TSA, Difco, Detroit, USA) amended with 50 μg cycloheximide/ml at

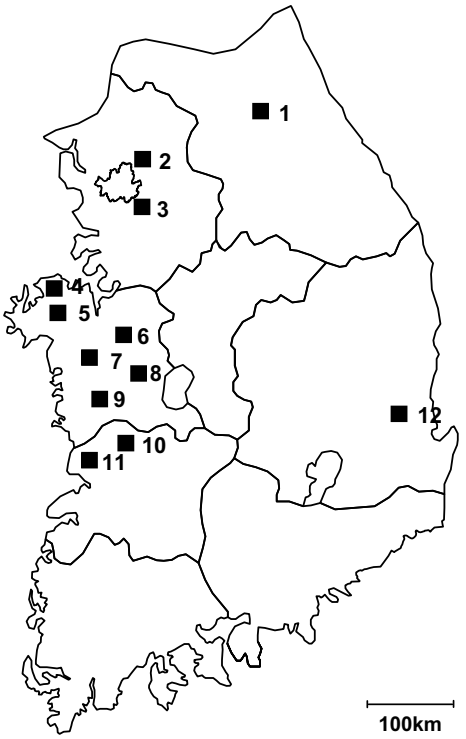

Fig. 1. Sampling locations for bacterial strains isolated from cucumber, pepper, and tomato plants grown in fields in Korea from 2001 to 2003. (1) Chuncheon, (2) Deokso, (3) Yongin, (4) Dangjin, (5) Seosan, (6) Yeasan, (7) Hongseong, (8) Gongju, (9) Buyeo, (10) Iksan, (11) Gunsan, and (12) Gyeongju.

Table 1
Sampling locations, sources and numbers of bacterial strains isolated from cucumber, pepper and tomato plants grown in the fields in Korea from 2001 to 2003

Location	Year collected	Source	Host	No of bacterial strain
Buyeo	2002	RI[a], RS, S	Pepper	8
Chuncheon	2003	RI, RS	Cucumber, Tomato	180
Dangjin	2002	RS, S	Cucumber	7
Deokso	2001	RI, RS, S	Pepper	13
Gongju	2002	RI, RS, S	Pepper	9
Gunsan	2002	RI, RS	Cucumber	16
Gyeongju	2003	RS	Pepper	76
Hongseong	2002	RS, S	Pepper	21
Iksan	2002	RI, RS, S	Cucumber	29
Seosan	2002	RI, RS, S	Cucumber	22
Yeasan	2002	RI, RS, S	Pepper	52
Yongin	2002	RI, RS, S	Pepper	6
Total				439

[a] Each bacterial strain was isolated from plant root interiors (RI), root surfaces (RS), or rhizosphere soils (S) of various plants.

28 °C for 48 h. Bacterial colonies with distinct morphologies were selected, streaked on nutrient agar (NA, Difco, Detroit, USA), and incubated at 28 °C for 48 h. Single colonies were cultured in nutrient broth (NB) in a rotary shaking incubator (160 rpm) at 28 °C for 24 h. Bacterial strains were then stored in NB amended with 20% glycerol at −70 °C until used.

For assays with radicles and seedlings of pepper (cv. Nockwang) susceptible to *P. capsici*, bacterial strains were streaked on NA and incubated at 28 °C for 48 h. The bacterial cells were harvested and suspended in 10 mM $MgSO_4$ buffer. The bacterial suspensions were adjusted to 10^8 bacterial cells/ml ($OD_{600} = 0.5$) with a spectrophotometer (Du® 650, Beckman Coulter, Inc., Fullerton, USA). For plant and field tests, single colonies were inoculated in 5 ml of NB and incubated in a shaking incubator (160 rpm) at 28 °C for 24 h. These pre-cultured bacteria were transferred into 500 ml of NB and incubated in a shaking incubator (160 rpm) at 28 °C for 48 h. The bacterial cells were harvested in the same buffer by centrifuging at 5000*g* at 20 °C for 15 min. After centrifugation, pellets (bacterial cells) were washed twice with 10 mM $MgSO_4$ buffer followed by centrifugation. Bacterial suspensions were adjusted to 10^8 bacterial cells/ml ($OD_{600} = 0.5$) in this buffer.

2.2. Radicle and seedling assays

Radicle and seedling assays with pepper seeds and seedlings were conducted as previously described (Chang et al., 2001; Kim et al., 2008). For the radicle assay, pepper seeds were germinated in petri dishes with moist filter papers (Whatman No. 1) for 3 days at 28 °C in the dark. Uniformly germinated seeds were soaked in the prepared bacterial suspension for 3 h and blotted on sterile filter papers. Ten seeds per bacterial strain were then placed at 28 °C on the margin of 5-day-old cultures of a virulent isolate of *P. capsici*, S197, on water agar amended with 0.02% glucose. The treated seeds were incubated at 28 °C with 16 h fluorescent light/day. Seeds treated with 10 mM $MgSO_4$ buffer served as controls. The incidence of infected radicles with brownish discoloration was determined 2–3 days after placing the seeds on water agar when all buffer-treated seeds (control) were completely infected.

For the seedling assay, pepper seeds germinated as described above were sown in 128-hole (3 × 3 × 5 cm) plug trays filled with potting mixture [peat moss (Acadian Peat Moss Ltd., Lamègue, New Brunswick, Canada) and TKS2 (Floragard Product, Oldenburg, Germany), 1:1.5, v/v] that included the prepared bacterial suspensions. The bacterial strains were incorporated at 10^8 bacterial cells/g potting mixture. Ten millimolar $MgSO_4$ buffer served as untreated control. The trays were placed at room temperature in a growth room with 16-h fluorescent light/day. After 2 weeks, pepper seedlings were exposed to 1 ml of *P. capsici* zoospore suspension (2000 zoospores/g potting mixture) at 1 cm below the soil-line near the seedlings. After inoculation, the seedlings were frequently watered for 2–3 days so that they would not dry out. Incidence of diseased seedlings was evaluated 4 days after inoculation. After the initial seedling assay of bacterial strains selected from the radicle assay, strains that exhibited significant ($P = 0.05$) difference compared to controls were selected for further tests. The selected strains from the radicle assay were tested two more times with three replications of eight seedlings each.

To prepare the pathogen inoculum as described by Kim et al. (1997), *P. capsici* S197 was grown on oatmeal agar for 7 days at 28 °C The cultures were then flooded with 20 ml of sterile distilled water and incubated under continuous fluorescent light for an additional 7 days at 28 °C to induce zoosporangia. Zoospores were released from zoosporangia when the cultures were flooded with chilled sterile water and stored at 4 °C for 30 min and then at room temperature for 30 min. Mycelia and sporangial debris were removed from the zoospores by filtration through three layers of cheesecloth. Zoospore concentration was determined by vigorously vortexing 1 ml of the zoospore suspension for 30 s to cause the zoospores to encyst, and the zoospores were then counted with a haemocytometer.

2.3. In planta trial

Sixteen bacterial strains selected from the seedling assay were evaluated in a growth room using 5-week-old pepper plants. Pepper seedlings, bacterial suspension, and pathogen inoculum were prepared as described above. Three weeks after seeding, seedlings were transplanted into 10-cm-diameter pots, which contained steam-sterilized soil, with saucers. One week later, the pots were drenched with 25 ml of the prepared bacterial suspensions. These plants were inoculated with *P. capsici* zoospores (25 zoospores/g soil) 1 week after the bacterial treatments. For inoculation, zoospore suspensions were injected into four holes (1 cm diameter × 1 cm deep) around each plant. Inoculated or uninoculated plants treated with 10 mM $MgSO_4$ buffer served as positive or negative controls, respectively. After inoculation, plants were continuously watered for 4–5 days through the saucers to prevent drying of the soil and then watered as needed. Disease severity was evaluated every day after initial disease appearance for 11–14 days after inoculation on a scale of 0 (symptomless) – 5 (plants dead) as described by Kim et al. (1989). Areas under disease progress curves (AUDPC) based on disease severity were also determined. This experiment was established in a completely random design and was conducted twice with 15 plants each.

2.4. Field tests

2.4.1. Drench treatment

Artificial inoculation field tests for drench treatment with five potential antagonistic bacterial strains (CCR04, CCR80, GSE09, ISE13, and ISE14) were conducted at

Deokso Experiment Farm of Korea University, Namyangju, Korea in 2005 and 2006. These bacterial strains were identified by analyses of 16S rDNA sequence (Fox et al., 1977), Biolog (Biolog, Hayward, USA), and fatty acid methyl-esters (Sasser, 1990) as well as transmission electron microscopy (Williams et al., 1989); strains CCR04 and CCR80 were identified as *Pseudomonas corrugata*, strain GSE09 as *Flavobacterium* sp., strain ISE13 as *Lysobacter enzymogenes*, and strain ISE14 as *Chryseobacterium indologenes*. These strains were preserved as *P. corrugata* CCR04 (KACC 91366P), *P. corrugata* CCR80 (KACC 91367P), *Flavobacterium* sp. GSE09 (KACC 91368P), *L. enzymogenes* ISE13 (KACC 91369P), and *C. indologenes* ISE14 (KACC 91370P) in the Korean Agricultural Culture Collection (KACC) of National Institute of Agricultural Biotechnology, Suwon, Korea.

Raised beds (20 cm high × 50 cm wide), spaced 85 cm apart (center to center), were constructed and covered with black plastic mulch. Pepper plants (cv. Nockwang) were grown for 9 weeks in 2005 and for 8 weeks in 2006 in pots containing commercial potting mixture (Sunshine® Mix Plug, Sun Gro Horticularue Canada Ltd., Seba Beach, Canada). These plants were transplanted into the beds in rows (30 cm between plants) on June 1, 2005 and May 10, 2006. Tests were established in beds with 12.5-m-long plots and arranged in a randomized complete block with four replications per treatment. Plots were treated with one of the antagonistic bacterial strains, 10 mM $MgSO_4$ buffer (untreated control), or metalaxyl (a.i. 7.5%, Ridomil MG®, Dongbu Hannong Chemicals Ltd., Seoul, Korea; fungicide-treated control). Bacterial suspensions for treatments were prepared as described above. Plants were drenched twice with 100 ml of each bacterial suspension or 10 mM $MgSO_4$ buffer per plant on July 23 and August 6 for the 2005 test, and on July 26 and August 2 for the 2006 test, respectively. Plants were treated once with 100 ml of metalaxyl (1 g per liter of water) according to the supplier's recommendation on August 6, 2005 and August 2, 2006 in each experimental year. Five days after the second bacterial treatment for each year, 10 ml of 10^4 zoospores/ml of *P. capsici*, prepared as described above, were inoculated into the soil around each plant. Evaluation of disease incidence was initiated when symptoms first appeared on the inoculated plants. The disease incidence was subsequently evaluated every 3 days for 27–30 days after inoculation.

2.4.2. Root-dip treatment

Natural inoculation field tests for root-dip treatment with the antagonistic bacteria were conducted at the same location in 2006 and 2007. Beds, plants and treatments were prepared as described above. However, 8 and 10-week-old plants were used and transplanted on May 10, 2006 and June 1, 2007, respectively. Tests were established in beds with 5 and 12.5 m-long plots and arranged in a randomized complete block with three and four replications per treatment for 2006 and 2007 tests, respectively. Plant roots were dipped in bacterial suspension or 10 mM $MgSO_4$ buffer just at transplanting into the beds in both experimental years; the metalaxyl (1 g per liter of water) treatment was only included in 2007. Final disease incidence (%) was evaluated on September 4, 2006 and September 18, 2007.

Pepper fruit production was also evaluated to assess the plant growth-promoting effect by the antagonistic bacteria in the 2007 test only. Marketable pepper fruits (over 8 cm long) were harvested three times on August 13, August 27, and September 22, 2007. At the first harvest, only ripe (red) fruits were collected and, at the second and third harvests, both green (unripe) and red (ripe) fruits were collected. Numbers and fresh weight (g) of fruits were determined with four replications per treatment with 40 plants each.

2.4.3. Root infection by P. capsici and populations of total bacteria and fungi

To assess root infections by *P. capsici*, roots of pepper plants drenched with the treatments were collected from the fields on September 10, 2005 and September 4, 2006; pepper roots dipped with the treatments were collected on September 4, 2006 and September 17, 2007. Four and five plants per treatment in a plot for the 2005 and 2006 drench tests, respectively, were randomly collected; five and three were collected for 2006 and 2007 root-dip tests. Soil attached to the root surfaces of the collected pepper plants was removed manually and roots were cut into 100 fragments (1 cm). These 100 fragments of plant root (three replicates per plant root) were placed on pimaricin–ampicillin–rifampicin–PCNB–hymexazol (PARPH) medium selective for *Phytophthora* spp. and incubated at 28 °C. Numbers of root fragments infected by *P. capsici* were evaluated 3–4 days after incubation.

The same soil samples collected for root infection assessment were used to evaluate populations of total bacteria and fungi. Ten grams of the rhizosphere soil of a plant (three replicates per plant) were placed into 100 ml of sterile water and shaken at 160 rpm at 28 °C for 30 min. The suspension was cultured at 28 °C for 48 h on TSA amended with 50 μg cycloheximide/ml and Ohio Agricultural Experiment Station (OAES) medium (Williams and Schmitthenner, 1960) for total bacteria and fungi, respectively. These experiments were conducted with the same replications used in the root infection assessment. Bacterial and fungal colonies were counted 2 and 3 days after incubation, respectively and assessed based on dry weight of soil.

2.5. Statistical analysis

Statistical analysis of data was conducted using the Statistical Analysis Systems (SAS Institute, Cary, USA). Percent data of disease incidence were statistically analyzed after arcsine square-root transformation. Analysis of variance was determined using the general linear model procedures, and means were separated with the least significant

difference (LSD). AUDPC was determined using the formula described by Shaner and Finney (1977) in which: $\text{AUDPC} = \sum_{i=1}^{n}(X_{i+1} + X_i)(t_{i+1} - t_i)/2$, where X_i = disease severity at the ith observation, t_i = time (day) at the ith observation, and n = total number of observations.

3. Results

3.1. Isolation of bacterial strains

A total of 439 bacterial strains (Table 1) were isolated from root interiors, root surfaces, or rhizosphere soils of cucumber, pepper, and tomato plants grown in fields in 12 locations in Korea from 2001 to 2003 (Fig. 1).

3.2. Sequential selection through radicle, seedling, and in planta tests

The 439 bacterial strains inhibited *P. capsici* infection on the radicles at various levels (Fig. 2). Out of 439 bacterial strains, 38 strains (8.4% of total strains) produced ⩽70% radicle infection (30% disease inhibition) by *P. capsici* compared to 100% infection in controls treated with $MgSO_4$ buffer. Among these 38 bacterial strains, two strains (0.5% of total strains) resulted in 40% radicle infection (60% disease inhibition); 11 strains (2.5%) 50% radicle infection (50%), 12 strains (2.7%) 60% radicle infection (40%), and 13 strains (3.0%) 70% radicle infection (30%). However, the remaining 401 strains (91.6%) provided little or no protection against pathogen infection on the radicles. In our previous study (Chang et al., 2001), we found that strains reducing *P. capsici* infection by more than 20% in radicle bioassay were significantly ($P = 0.05$) antagonistic.

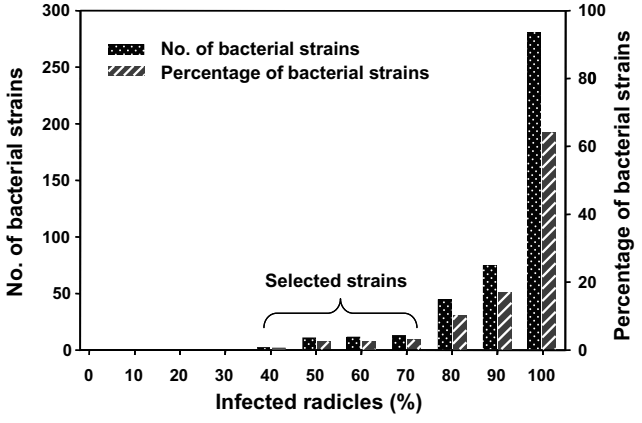

Fig. 2. Numbers and percentage of bacterial strains among the 439 strains tested in a radicle assay using pepper seeds (cv. Nockwang) for radicle infection by *Phytophthora capsici*. In the radicle assay, germinated seeds treated with 10 mM $MgSO_4$ buffer (control, untreated) or bacterial strains (10^8 bacterial cells/ml) for 3 h were placed onto the margin of 5-day-old cultures of *P. capsici* on water agar containing 0.02% glucose. Incidence (%) of radicles infected by *P. capsici* on seeds treated with the bacterial strains was determined 2–3 days after treatments when all control seeds were completely infected. The experiment was conducted with three replications of 10 seeds each per treatment.

However, we selected strains with at least 30% radicle protection to increase the probability of obtaining better biocontrol agents.

An initial seedling assay was conducted using the 38 strains selected from the radicle assay (Fig. 2); consequently, 16 potentially antagonistic strains (data not shown) were selected based on significant ($P = 0.05$) difference compared to the buffer-treated control. These 16 strains were then used to examine whether disease suppression was expressed consistently in both seedling assays and *in planta* trials (Table 2). In seedling assays, disease incidence ranged from 21 to 55%. All tested bacterial strains significantly ($P = 0.05$) suppressed disease incidence compared to the buffer-treated controls. In the *in planta* trial using 5-week-old pepper plants, all tested bacterial strains significantly ($P = 0.05$) and consistently reduced both final disease severity and AUDPC compared to the buffer-treated controls (Table 2). Among these 16 strains, five bacterial strains, CCR04, CCR80, GSE09, ISE13, and ISE14,

Table 2
Disease incidence (%) and severity, and areas under the disease progress curves (AUDPC) caused by *Phytophthora capsici* on pepper (cv. Nockwang) seedlings and plants treated with 16 potentially antagonistic bacterial strains selected from the radicle assay

Treatment[a,b]	Seedling assay[c] (disease incidence)	*In planta* trial[d]	
		Disease severity	AUDPC
Untreated	95.8 a	4.1 a	18.6 a
CCR01	52.1 bc	3.1 bc	12.6 bc
CCR04	21.1 g	1.6 gh	4.5 h
CCR10	52.1 bc	3.2 b	14.8 b
CCR40	29.5 fg	2.2 d–g	7.0 e–h
CCR43	27.4 fg	1.4 h	4.3 h
CCR48	35.1 d–f	1.6 gh	5.8 f–h
CCR55	55.1 b	2.4 d–f	10.1 c–e
CCR80	32.2 e–g	1.7 f–h	5.2 gh
GSE06	36.2 c–f	2.3 d–f	8.4 d–g
GSE07	49.1 b–d	2.7 b–d	12.5 bc
GSE09	29.5 fg	1.8 e–h	5.8 f–h
GSE10	40.8 b–f	2.7 b–d	11.4 cd
ISE13	31.8 e–g	1.8 f–h	6.1 f–h
ISE14	37.5 c–f	1.9 e–h	8.6 d–f
SSS08	47.0 b–e	2.7 b–d	10.6 cd
YSE20	36.3 c–f	2.5 c–e	8.6 d–f

[a] Means within a column followed by the same letter are not significantly different when tested with LSD at $P = 0.05$. For disease incidence, arcsine square root-transformed data were used for statistical analyses; however, untransformed data are presented.

[b] Plants were treated with 10 mM $MgSO_4$ buffer (untreated control) or bacterial cells (10^8 bacterial cells/ml).

[c] Two-week-old pepper seedlings in a potting mixture incorporated with the bacterial strains were inoculated with 2×10^3 zoospores of *P. capsici* per gram potting mixture. Disease incidence (%) of the seedlings was determined 4 days after inoculation. Values are means of six replications with eight seedlings each from combined data of two experiments.

[d] Five-week-old pepper plants were inoculated with 25 zoospores per gram soil. Disease severity was evaluated on a scale of 0 (symptomless)–5 (plants dead) 11 and 14 days after inoculation in two experiments. AUDPC was determined based on disease severity for eight and 10 observations over 11 and 14 days after inoculation in two experiments, respectively. Values are means of 30 replications from combined data of two experiments.

were selected as potentially effective antagonists for further field tests. These five strains were selected considering their field source and origins from root surfaces (strains CCR04 and CCR80) and root interiors (strains GSE09, ISE13, and ISE14) as well as their consistent ability to suppress disease.

3.3. Field tests

3.3.1. Drench treatment

Phytophthora blight of pepper was severe in the fields in both years and the final disease incidence ranged from 14 to 65% in 2005 and 16 to 69% in 2006 (Fig. 3). In the 2005 test, all five potentially antagonistic strains produced significantly ($P = 0.05$) lower disease incidence than buffer-treated control 30 days after inoculation. However, for strain GSE09 disease incidence was only significantly different from that of the control at the final observation. In the 2006 test, four strains, CCR04, CCR80, GSE09, and ISE14, significantly ($P = 0.05$) reduced disease incidence compared to the buffer-treated control over 27 days after inoculation; however, strain ISE13 failed to reduce the disease. Metalaxyl produced one of the lowest disease incidences among the treatments in both years (Fig. 3).

Rate of *P. capsici*-root infection in response to treatments corresponded with disease incidence as evaluated in the field (Fig. 4). In the 2005 test, four strains, except strain GSE09, significantly ($P = 0.05$) reduced root infection compared to the buffer-treated control. Similar results were obtained in the 2006 test; four strains, except strain ISE13, significantly ($P = 0.05$) reduced root infection. Metalaxyl also produced one of the lowest root infections by *P. capsici* in both years (Fig. 4). When numbers of total bacteria and fungi in rhizosphere soils of plants drenched bacterial strains were compared, total bacteria per g soil ranged from $\log_{10}$ 6.50 to 6.79 in 2005 and $\log_{10}$ 5.93 to 6.27 in 2006, while total fungi ranged from $\log_{10}$ 4.31 to 4.54 in 2005 and $\log_{10}$ 3.23 to 3.40 in 2006 (Table 3). Total bacteria and fungi in bacterial strain-treated plants were not significantly ($P = 0.05$) different from the buffer and fungicide-treated controls in either year (Table 3).

3.3.2. Root-dip treatment

Phytophthora blight of pepper plants was severe in the fields, and the final disease incidence ranged from 11 to 87% in 2006 and 23 to 65% in 2007 (Table 4). In the 2006 test, four strains, CCR04, CCR80, GSE09, and ISE14 significantly ($P = 0.05$) reduced disease incidence compared to the buffer-treated control, but strain ISE13 failed to reduce the disease. In the 2007 test, all five strains significantly inhibited disease incidence compared to the buffer-treated control. Metalaxyl-dipped plants also

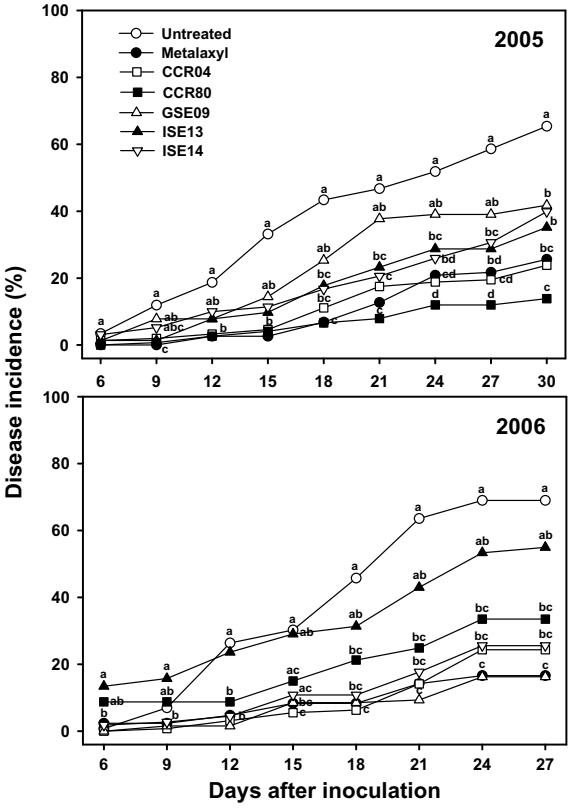

Fig. 3. Progress curves for incidence of Phytophthora blight of pepper plants (cv. Nockwang) drenched with five candidate antagonistic bacterial strains, CCR04, CCR80, GSE09, ISE13, and ISE14, 10 mM $MgSO_4$ buffer (untreated control), or metalaxyl (fungicide-treated control) under artificially inoculated field conditions at Deokso Experiment Farm of Korea University, Namyangju, Korea in 2005 and 2006. Means followed by the same letter are not significantly different when tested with LSD at $P = 0.05$. For disease incidence, arcsine square-root transformed data were used for statistical analyses; however, untransformed data are presented.

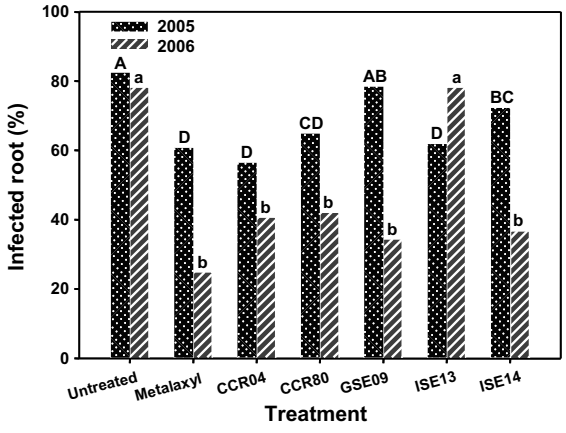

Fig. 4. *Phytophthora capsici*-infected roots of pepper plants that were drenched with antagonistic bacterial strains, 10 mM $MgSO_4$ buffer (untreated control), or metalaxyl (fungicide-treated control) just prior to transplanting under artificially inoculated field conditions in 2005 and 2006. Percentage of infected plant roots was determined on the PARPH medium as follows: number of infected root fragments (1 cm)/total number of root fragments × 100%. Different capital or small letters on bars indicate significant difference when tested with LSD at $P = 0.05$ in a test year. Arcsine square root-transformed data were used for statistical analyses; however, untransformed data are presented.

Table 3
Total bacteria and fungi in rhizosphere soils of pepper plants drenched with various antagonistic bacterial strains and metalaxyl under artificially inoculated field conditions in 2005 and 2006

Treatment[a,b]	Total bacteria[c] (Log_{10} CFU/g dry wt. soil)		Total fungi (Log_{10} CFU/g dry wt. soil)	
	2005	2006	2005	2006
Untreated	6.62 ± 0.11a–c	6.23 ± 0.16a	4.45 ± 0.05ab	3.38 ± 0.01a
Metalaxyl	6.52 ± 0.05bc	6.00 ± 0.08a	4.31 ± 0.08b	3.27 ± 0.06a
CCR04	6.79 ± 0.03a	6.06 ± 0.09a	4.51 ± 0.07a	3.32 ± 0.04a
CCR80	6.57 ± 0.03bc	6.10 ± 0.08a	4.48 ± 0.03a	3.33 ± 0.00a
GSE09	6.70 ± 0.08ab	6.20 ± 0.09a	4.50 ± 0.03a	3.23 ± 0.06a
ISE13	6.64 ± 0.03a–c	6.27 ± 0.04a	4.54 ± 0.03a	3.40 ± 0.02a
ISE14	6.50 ± 0.06c	5.93 ± 0.08a	4.49 ± 0.03a	3.28 ± 0.07a

[a] Means ± standard errors within a column followed by the same letter are not significantly different when tested with LSD at $P = 0.05$.
[b] Plants were treated twice with bacterial strains or 10 mM $MgSO_4$ buffer (untreated control) on July 23 and August 6 for the 2005 test, and on July 26 and August 2 for the 2006 test. Plants were also treated once with metalaxyl (fungicide-treated control) on August 6, 2005 and August 2, 2006. The plants were transplanted into the beds on June 1, 2005 and May 10, 2006.
[c] Total bacteria and fungi were determined on TSA medium amended with cycloheximide and OAES medium, respectively. These experiments were conducted with four replications of four and five plants each in 2005 and 2006, respectively.

Table 4
Disease incidence and *Phytophthora capsici*-infected roots in pepper plants that were root-dipped with various antagonistic bacterial strains and metalaxyl just prior to transplanting in the field with natural inoculum in 2006 and 2007

Treatment[a,b]	Disease incidence (%)[c]		Infected root (%)[d]	
	2006	2007	2006	2007
Untreated	87.3 ± 5.6 a	65.3 ± 2.3a	86.0 ± 2.8a	65.7 ± 2.9a
Metalaxyl	—[e]	22.5 ± 3.1c	—	40.5 ± 8.8b
CCR04	36.7 ± 2.4b	33.8 ± 6.8bc	49.3 ± 1.8b	39.5 ± 5.8b
CCR80	11.4 ± 4.5b	30.6 ± 1.9bc	40.9 ± 6.1b	41.2 ± 1.7b
GSE09	19.6 ± 8.9b	33.8 ± 2.2b	51.2 ± 5.9b	48.2 ± 4.8b
ISE13	78.6 ± 15.2a	38.8 ± 2.6b	70.8 ± 0.3a	46.6 ± 2.4b
ISE14	25.5 ± 8.6b	38.3 ± 2.6b	24.2 ± 2.9c	46.6 ± 1.6b

[a] Means ± standard errors within a column followed by the same letter are not significantly different when tested with LSD at $P = 0.05$.
[b] Plants were treated with antagonistic bacterial strains, 10 mM $MgSO_4$ buffer (untreated control), or metalaxyl (fungicide-treated control) on May 10 and June 1 for 2006 and 2007 tests.
[c] Disease incidence was evaluated on September 4, 2006 and September 18, 2007. These experiments were conducted with three replications of 15 plants each in 2006 and four replications of 40 plants each in 2007.
[d] Percentage of infected plant roots (%) was determined on the PARPH medium as follows: number of infected root fragments (1 cm)/total number of root fragments × 100%. These experiments were conducted with three replications of five plants each in 2006 and four replications of three plants each in 2007.
[e] —, not tested.

produced one of the lowest disease incidences among the treatments in the 2007 test (Table 4). Similar results were obtained from pepper root infection by *P. capsici*, in which all tested bacterial strains, except strain ISE13, significantly ($P = 0.05$) reduced root infection in 2006, while all strains inhibited pathogen infection in 2007. Metalaxyl also

Table 5
Total bacteria and fungi in rhizosphere soils of pepper plants root-dipped with various antagonistic bacterial strains and metalaxyl just prior to transplanting in the field with natural inoculum in 2006 and 2007

Treatment[a,b]	Total bacteria[c] (Log_{10} CFU/g dry wt. soil)		Total fungi (Log_{10} CFU/g dry wt. soil)	
	2006	2007	2006	2007
Untreated	5.72 ± 0.11a	6.46 ± 0.12a	3.08 ± 0.07a	3.12 ± 0.10a
Metalaxyl	—[d]	6.63 ± 0.14a	—	3.30 ± 0.17a
CCR04	5.92 ± 0.16a	6.65 ± 0.18a	3.11 ± 0.17a	3.29 ± 0.09a
CCR80	5.73 ± 0.09a	6.69 ± 0.12a	3.19 ± 0.15a	3.23 ± 0.13a
GSE09	5.69 ± 0.11a	6.48 ± 0.09a	3.08 ± 0.07a	3.19 ± 0.05a
ISE13	5.98 ± 0.12a	6.56 ± 0.11a	3.17 ± 0.03a	3.30 ± 0.12a
ISE14	5.75 ± 0.12a	6.56 ± 0.09a	3.15 ± 0.09a	3.08 ± 0.04a

[a] Means ± standard errors within a column followed by the same letter are not significantly different when tested with LSD at $P = 0.05$.
[b] Plants were treated with bacterial strains, 10 mM $MgSO_4$ buffer (untreated control), or metalaxyl (fungicide-treated control) on May 10 and June 1 for 2006 and 2007 tests.
[c] Total bacteria and fungi were determined on TSA medium amended with cycloheximide and OAES medium, respectively. These experiments were conducted with three replications of five plants each in 2006 and four replications of three plants each in 2007.
[d] —, not tested.

produced less infected roots among the treatments in 2007 (Table 4). When numbers of total bacteria and fungi in rhizosphere soils of the root-dipped plants were compared, total bacteria per gram soil ranged from log_{10} 5.69 to 5.98 in 2006 and log_{10} 6.46 to 6.69 in 2007, while total fungi ranged from log_{10} 3.08 to 3.19 in 2006 and log_{10} 3.08 to 3.30 in 2007 (Table 5). Total bacteria and fungi in bacterial strain-treated plants were not significantly ($P = 0.05$) different from the buffer and fungicide-treated controls in either years (Table 5).

When numbers and weights of pepper fruits of root-dipped plants with the bacterial strains were compared in 2007 alone, strains CCR80, ISE13 and ISE14 significantly ($P = 0.05$) increased numbers and/or weights of green (unripe) and/or red (ripe) pepper fruits compared to the buffer-treated controls (Table 6). However, strains CCR04 and GSE09 and metalaxyl failed to increase the amounts of pepper fruits compared to the buffer-treated control (Table 6).

4. Discussion

Recently, we developed a sequential screening procedure that includes radicle and seedling assays and *in planta* trials to select potentially antagonistic bacteria to control Phytophthora blight of pepper (Kim et al., 2008). Using this procedure, we obtained 16 potentially antagonistic bacterial strains from 439 strains and selected five final candidate strains. In this study, we evaluated the control efficacy of drench and root-dip treatments of the selected candidate antagonistic strains against Phytophthora blight of pepper in the field with artificial and natural inoculations.

In 2005 and 2006 tests, the control efficacy of five candidate bacterial strains, CCR04, CCR80, GSE09, ISE13, and

Table 6
Numbers and fresh weights of pepper fruits harvested from plants root-dipped with various antagonistic bacterial strains and metalaxyl just prior to transplanting in the field with natural inoculum in 2007

Treatment[a,b]	Number of pepper fruit[c]			Fresh weight of pepper fruit (g)		
	Green	Red	Combined	Green	Red	Combined
Untreated	287 ± 14 c	150 ± 8 b	437 ± 16 c	2423 ± 215 a	1728 ± 124 b	4151 ± 216 c
Metalaxyl	324 ± 37 a–c	138 ± 16 b	462 ± 46 bc	2621 ± 391 a	1674 ± 234 b	4295 ± 585 bc
CCR04	297 ± 34 bc	177 ± 25 ab	473 ± 55 bc	2381 ± 277 a	2200 ± 296 ab	4581 ± 541 a–c
CCR80	433 ± 36 a	221 ± 8 a	654 ± 32 a	3480 ± 314 a	2646 ± 118 a	6126 ± 386 ab
GSE09	318 ± 31 a–c	167 ± 8 ab	485 ± 38 a–c	2747 ± 137 a	1971 ± 107 ab	4718 ± 205 a–c
ISE13	421 ± 57 ab	168 ± 20 ab	588 ± 70 a–c	3364 ± 555 a	1984 ± 227 ab	5348 ± 718 a–c
ISE14	395 ± 60 a–c	220 ± 37 a	616 ± 86 ab	3417 ± 557 a	2761 ± 575 a	6178 ± 1076 a

[a] Means ± standard errors within a column followed by the same letter are not significantly different when tested with LSD at $P = 0.05$.
[b] Plants were treated with bacterial strains, 10 mM $MgSO_4$ buffer (untreated control), or metalaxyl (fungicide-treated control) on June 1, 2007.
[c] Number and fresh weights (g) of pepper fruits per replication were described with total fruit numbers and weights from three harvests on August 13, 27, and September 22, 2007. At the first harvest, only red (ripe) pepper fruits were collected and at the second and third harvests, red and green (unripe) pepper fruits were collected. These experiments were conducted with four replications of 40 plants each.

ISE14, was examined against Phytophthora blight of pepper plants drenched with the bacterial suspensions in the field with artificial inoculation of *P. capsici*. Three out of five strains, CCR04, CCR80, and ISE14, consistently provided effective control of the soilborne disease compared to untreated controls in both tests. However, strain GSE09 failed to provide disease reduction in the 2005 test and strain ISE13 in the 2006 test. It could be that the drench treatment did not sufficiently cover the root systems in these tests, thus not allowing the treatment to protect the infection courts of the roots against *P. capsici* infection. This speculation could be supported by the observations that pepper roots drenched with these bacterial suspensions in these particular tests had similar infection rates by the pathogen compared with roots treated with the buffer.

Protection from *P. capsici* infection during the early stages of pepper plant development is important since pepper seeds and seedlings are susceptible to the pathogen (Hwang and Kim, 1995). Pepper seedlings grown for several weeks in soilless mixes in a greenhouse are routinely transplanted into the fields in Korea. Antagonistic bacteria often display their disease suppressive ability uniformly in soilless mixes (Spadaro and Gullino, 2005). Therefore, we applied the candidate bacterial strains to pepper seedlings in potting mixes just prior to transplanting into the field with natural inoculum. This root-dip treatment could save labor and time in applying bacterial suspensions compared to the drench treatment in the field. In 2006 and 2007 tests, four out of five bacterial strains CCR04, CCR80, GSE09, and ISE14 showed consistently good control efficacy against *P. capsici* infection in the field. The strain GSE09, which did not inhibit disease development consistently in the drench treatment tests, generated consistent and effective disease reduction in the root-dip treatments in both 2006 and 2007 tests. However, strain ISE13 failed again to reduce disease in the 2006 test as observed in the drench treatment tests. Similarly, strain ISE13 did not inhibit root infection rate by the pathogen compared to the untreated controls.

Strains CCR80 and ISE14 increased production of pepper fruits, and especially ripe red fruits, when the roots of pepper seedlings were dipped in these suspensions just prior to transplanting in the field; this result indicates that these strains could have plant growth-promoting effects. Strain CCR80 increased pepper fruit weights in our previous small plot field tests for root-dip treatments with artificial inoculation (Sang et al., 2007). Since metalaxyl treatment (fungicide-treated control) did not differ from the untreated control in terms of pepper production, there is little possibility that the increased fruit production was due to disease suppression alone by the antagonistic strains. Furthermore, these strains might stimulate ripening of pepper fruits since they significantly increased the ripe red fruits, but not the unripe green fruits. This may result from alterations in levels of signal transduction compounds or hormones such as ethylene, IAA, ascorbic acid, and jasmonate, or production of volatile compounds by the antagonistic bacterial strains (Ryu et al., 2003; Ping and Boland, 2004; Orhan et al., 2006; Mena-Violante and Olalde-Portugal, 2007). From the overall results of drench and root-dip treatment tests, strains CCR04, CCR80, and ISE14 could adequately protect pepper plants from Phytophthora blight of pepper throughout plant development regardless of drench or root-dip treatment in the field.

The biocontrol efficacy of the candidate bacterial strains against Phytophthora blight of pepper by drench or root-dip treatments might be due to continuous colonization of the strains with little influence of total bacteria and fungi in pepper rhizospheres under field conditions. Root colonization by these strains has been frequently observed in our preliminary laboratory tests (our unpublished data) although colonization has not been evaluated in the field tests. Colonization could also give the strains an advantageous niche for utilizing root exudates such as sugars, organic acids and amino acids as nutrients (Lugtenberg et al., 1999; Bolwerk et al., 2003; Kamilova et al., 2006). Consequently, continuous colonization might protect root infection courts from the soilborne pathogen *P. capsici*.

In the case of strain ISE13, the strain protected plants from infection only in one year's test and its ability for continuous colonization in pepper roots might be limited due to effects of subtle environmental changes in the field. This strain has the ability to produce antibiotics against *P. capsici* (our unpublished data) and other plant pathogens in media (Sang et al., 2006). Its antibiotic production in the rhizosphere, as one of the factors for disease suppression, might be affected by diverse environmental and biological factors such as temperature, deleterious or indigenous microorganisms, or nutrients under field conditions (Hase et al., 1999; Gu and Mazzola, 2001; Landa et al., 2004; Li and Kremer R.J., 2006). This might be another reason for its inconsistent protection against pathogen infection in repeated field tests.

In conclusion, the antagonistic bacterial strains selected through the sequential screening procedure appeared to provide significant protection against the soilborne oomycete pathogen, *P. capsici*, through pepper root colonization. These strains could be applied by either drench or root-dip treatment for agricultural use and are biocontrol agents as agricultural alternatives for controlling Phytophthora blight of pepper.

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