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Biocontrol of Late Blight (*Phytophthora capsici*) Disease and Growth Promotion of Pepper by *Burkholderia cepacia* MPC-7

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A chitinolytic bacterial strain having strong antifungal activity was isolated and identified as *Burkholderia cepacia* MPC-7 based on 16S rRNA gene analysis. MPC-7 solubilized insoluble phosphorous in hydroxyapatite agar media. It produced gluconic acid and 2-ketogluconic acid related to the decrease in pH of broth culture. The antagonist produced benzoic acid (BA) and phenylacetic acid (PA). The authentic compounds, BA and PA, showed a broad spectrum of antimicrobial activity against yeast, several bacterial and fungal pathogens *in vitro*. To demonstrate the biocontrol efficiency of MPC-7 on late blight disease caused by *Phytophthora capsici*, pepper plants in pot trials were treated with modified medium only (M), M plus zoospore inoculation (MP), MPC-7 cultured broth (B) and B plus zoospore inoculation (BP). With the sudden increase in root mortality, plants in MP wilted as early as five days after pathogen inoculation. However, plant in BP did not show any symptom of wilting until five days. Root mortality in BP was markedly reduced for as much as 50%. Plants in B had higher dry weight, P concentration in root, and larger leaf area compared to those in M and MP. These results suggested that *B. cepacia* MPC-7 should be considered as a candidate for the biological fertilizer as well as antimicrobial agent for pepper plants.

Keywords : antimicrobial activity, biological fertilizer, phosphate solubilization, root mortality, zoospore

Pepper (*Capsicum annuum* L.) is an important vegetable crop in Korea (Chae et al., 2005). *Phytophthora* blight of pepper, which is caused by *Phytophthora capsici* is one of the most devastating soilborne diseases of pepper in Korea

(Hwang and Kim, 1995), India, United States and other pepper growing areas worldwide (Babu et al., 2011). This soilborne pathogen can infect all parts of the pepper plant via water splashing from the soil to the foliage and can rapidly disperse in surface water (Ristaino and Johnston, 1999). The chemical and cultural measures cannot always control persistent propagules such as *P. capsici* oospores (Lamour and Hausbeck, 2003). Moreover, their efficiency is decreased due to evolution of resistance by the pathogen (Rosenberger and Meyer, 1981) and the use of synthetic chemicals can also kill useful soil insects and beneficial microorganisms in the rhizosphere (Bartlett et al., 2002).

Several species of bacteria are known to have beneficial effect on plant growth and disease suppression through the production of plant growth-promoting regulators and antibiotic substances (Ren et al., 2011). One of the direct mechanisms that promote plant growth by the plant growth promoting rhizobacteria (PGPR) includes the provision of bioavailable nutrient for plant uptake (Richardson, 2001). On the other hand, indirect growth promotion occurs through the decrease or prevention of deleterious effect of pathogenic microorganisms, mostly due to antagonism (Ortega-Morales et al., 2009) and the synthesis of antibiotics (Sivan et al., 1992).

Supplying P for plant uptake through biological means is a viable alternative (Kim et al., 1998). Solubilization of phosphorus in rhizosphere is the most common mode of action implicated in PGPR that increase the nutrient availability to the host plant (Richardson, 2001). Particularly, phosphate-solubilizing bacteria (PSB) solubilize the insoluble inorganic P by producing organic acids (Leyval and Berthelin, 1989). These organic acids can either directly dissolve the mineral phosphates as a result of anion exchange or can chelate both Fe and Al ions associated with phosphate (Bajpai and Rao, 1971). The organic acids involved in phosphate solubilization include oxalic, citric, butyric, malonic, lactic, succinic, malic, gluconic, acetic,

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glyconic, fumaric, adipic, and 2-ketogluconic acid (Kim et al., 1998; Sperber, 1957). Among them, gluconic acid seems to be the most frequent agent for mineral P solubilization (Illmer and Schimmer, 1992) and it is produced by direct oxidation of glucose via membrane-bound quinoprotein GDH enzyme (Patel et al., 2008). In addition, Moghimi et al. (1978) reported that 2-ketogluconic acid was the only organic acid present in significant amounts, representing about 20 per cent of the rhizosphere products. The microbial simulated P solubilization simultaneously increases the P uptake and yield in many crops as demonstrated in tomato, potato, rice, sugar beet and citrus with *Pseudomonas fluorescens* (Kloepper et al., 1988), potato with *Bacillus* spp. (Burr et al., 1978), and wheat with *Azospirillum* spp. (Baldani et al., 1986).

Antibiotics to pathogen growth suppression have been isolated from a number of bacterial species such as *Burkholderia ambifaria* (Li et al., 2002) and *Pseudomonas* spp. (Paul and Sarma, 2006). The distinguished antibiotic productions such as pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol by *Pseudomonas fluorescens* Pf-5 (Loper et al., 2007) and various antifungal lipopeptides by *Bacillus amyloliquefaciens* FZB42 (Koumoutsis et al., 2004) have been previously reported. Weak acids such as benzoic acid are used to preserve foods and beverages. Once they enter the neutral pH of the cytoplasm, they dissociate to release an anion and a proton. The pH gradient across the plasma membrane drives the intracellular accumulation of protons and anions, reducing the intracellular pH and inhibiting the growth of the micro-organism (Booth and Kroll, 1989).

In this experiment, we focused to isolate a multifunctional strain producing organic acids and antimicrobial compounds during bacterial growth and tested its practical application on pepper in pot trial. Therefore, the objectives of this investigation were to isolate a strain that had both strong phosphate solubilizing and disease suppressing activities; to extract and identify the antimicrobial compounds; to determine the biological activities of isolated compounds *in vitro*, and to investigate the biocontrol efficiency of the new isolate on *Phytophthora* blight disease on pepper *in vivo*.

Materials and Methods

Isolation of bacterial strain. Soil samples collected from pepper fields of Naju area, Korea, were serially diluted with sterile water up to 10^{-6} times and 100 μ l of soil suspension was spread on the surface of chitin media (CM) (wt/vol; chitin 0.5%; Na_2HPO_4 0.2%; KH_2PO_4 0.1%; NaCl 0.05%; NH_4Cl 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.05%; KNO_3 0.05%; yeast extract 0.01%; and agar 2%; pH 7).

After five days incubation at 30°C, the strains which showed the halo zones were selected and checked for the antifungal activity against *Phytophthora capsici*, *Rhizoctonia solani* AG-1 (IA), and *Fusarium oxysporum* f. sp. *lycopesci* by dual culturing method on the CP media which was composed of 50% of chitin media (CM) and 50% of potato dextrose agar media (PDA). Finally, the strongest strain against all three fungal pathogens was selected. The bacterium was cultured in a 500-ml Erlenmeyer flask containing 200 ml LB broth (Luria Bertani, Detroit, MI) and incubated for 5 days at 28°C on a rotary shaker. The broth culture was then cryopreserved at -75°C in 50% (vol/vol) glycerin until further use (Sopheareth et al., 2006).

Identification of bacterial strain. The 16S rRNA gene analyses were performed directly using a single colony of the strain MPC-7 isolate, according to the procedure described by Di Cello (1997). The forward primer was 5'-TGGCTCAGAAGCAACGCTGGCGGC-3', and the reverse primer was 5'-CCCCTGCTGCCTCCCGIAGGAGT-3'. The temperature cycle was at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min 30 s for 30 cycles and 5 min at 72°C for extension. The polymerase chain reaction product was cloned using pGEM-T easy vector (Promega, Madison, WI). The nucleotide sequence of the 16S rRNA gene was determined by Biodye Terminator cycle sequencing kit and compared with published 16S rRNA sequences using Blast search at Gene Bank Data base of NCBI (Bethesda, MD).

Phosphate solubilization and measurement of organic acid produced by *B. cepacia* MPC-7. The selected strain was checked for its phosphate solubilizing activity on hydroxyapatite agar (HA) medium [wt/vol; $\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6$ 0.4%; NaCl 0.1%; $\text{MgSO}_4 \cdot 2\text{H}_2\text{O}$ 0.04%; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.02%; KCl 0.02%; yeast extract 0.02%; yeast autolysate 0.02%; tryptone peptone 0.02% and agar 1.5%, pH 7]. After incubation at 30°C for three days, the appearance of clear zone around the colony was observed.

For the measurement of organic acid production, the selected strain was grown in culture broth (CB) containing colloidal chitin 0.5%; hydroxyapatite 0.4%; glucose 1%; NH_4Cl 0.1%; NaCl 0.05%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.05%; KNO_3 0.05%; yeast extract 0.01% and pH 7.0 at 30°C and 170 rpm for 5 days. Broth culture was filtered through a Whatman 0.2 μ m membrane filter. Organic acids in filtrate were determined by high performance liquid chromatography (HPLC) (Shimadzu, Japan) with Shodex RSpak KC-811 column (8 mm \times 30 cm). The operating conditions consisted of 0.1% H_3PO_4 as the mobile phase, a constant flow rate of 0.5 ml min^{-1} , and sample injection of 20 μ l under the UV 210 nm. The organic acids were quantitatively determined by comparing peak areas of

chromatograms with those of authentic standards.

Measurement of changes in pH and P concentration of the culture media. The pH and P concentrations in the same culture broth (CB) were also measured every 24 h. The pH of broth culture was measured directly by immersing the probe of pH meter into culture solution. To measure P concentration, the supernatant was filtered through Whatman No.6 filter paper. Fifty microliter of filtrate was poured into a 20 ml test tube containing 1.95 ml extracting solution. Then, 5 ml of distilled water, 2 ml of 12 mM ammonium paramolybdate solution and 1 ml of 5 mM SnCl₂ were added and mixed well. After incubation for 5 minutes, the concentration of P₂O₅ was measured spectrophotometrically at 660 nm (Olsen and Sommers 1982).

Extraction, identification and elucidation of antimicrobial compounds. MPC-7 was grown in CB at 30°C on a rotary shaker for 96 h. The culture broth was centrifuged at 6000 rpm for 10 min at 4°C. The supernatant was acidified with 1 N HCl to pH 3 and extracted with an equal volume of ethyl acetate (EtOAc, 15-L three times). The EtOAc soluble organic fraction was concentrated under vacuum using a rotary evaporator (Büchi, Switzerland). Then, the ethyl-acetate soluble organic fraction (0.4 g) was loaded on

Sephadex LH-20 column chromatography. The active fraction (102 mg) was further purified by octadecylsilane (ODS) column chromatography with MeOH-H₂O as stepwise and an active fraction was dissolved at 60% MeOH (5.4 mg). Finally, two active peaks from HPLC analysis were further identified by GC-EI-MS.

Microorganisms. Bacterial and fungal pathogens used in this assay were purchased from Korean Agricultural Culture Collection (KACC) and Korean Collection for Type Cultures (KCTC) in Suwon, South Korea (Table 1).

Bactericidal activity assay. The antimicrobial assay was conducted on sterile petri plates (91 mm) containing 20 ml of 1.5% nutrient agar (Lam et al., 2000). An aliquot of 50 µl of bacterial inoculum containing 10⁸ cfu ml⁻¹ was added to 20 ml of nutrient broth medium plus 1.5% Bacto agar cooled at 45°C. This medium was then poured into petri dishes, and maintained for 1 h at room temperature. Sterile paper discs were loaded with 10 µl of each benzoic acid and phenylacetic acid purchased from Sigma-Aldrich Chemical Company in methanol dilutions to a known concentration (500 ppm). After drying, six discs including one methanol-impregnated negative control were placed on the surface of each agar plate. A transparent ring around the paper disk

Table 1. The inhibition diameter of 500 ppm benzoic acid (BA) and phenylacetic acid (PA) against yeast and plant pathogenic bacteria after 24 h of incubation at 30°C or 37°C and the degree of inhibition against filamentous fungi 5 days after incubation at 26°C

Microorganisms	Strain Identification	Growth Inhibition	
		BA	PA
Bacteria			
<i>Agrobacterium tumefaciens</i>	KACC ^a 10298	15.2 ± 0.2	15.3 ± 0.2*
<i>Escherichia coli</i>	KCTC ^b 2593	13.7 ± 0.2	15.7 ± 0.3
<i>Micrococcus luteus</i>	KCTC 3523	11.2 ± 0.2	11.0 ± 0.3
<i>Pseudomonas aeruginosa</i>	KCTC 2513	12.5 ± 0.3	12.3 ± 0.2
<i>Staphylococcus aureus</i>	KCTC 1928	13.0 ± 0.3	12.3 ± 0.2
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>	KACC 11157	17.3 ± 0.3	16.2 ± 0.2
Yeast			
<i>Saccharomyces cerevisia</i>	KACC 7904	13.7 ± 0.2	11.2 ± 0.2
Fungi			
<i>Alternaria brassicicola</i>	KACC 40034	±	±
<i>Botrytis cinerea</i>	KACC 40573	++	±
<i>Didymella bryoniae</i>	KACC 40900	++	+
<i>Fusarium oxysporum</i> f. sp. <i>gladioli</i>	KACC 40051	++	±
<i>Fusarium oxysporum</i> f. sp. <i>melonis</i>	KACC 40904	+	-
<i>Phytophthora capsici</i>	KACC 40483	++	++
<i>Pythium aphanidermatum</i>	KACC 40156	++	++
<i>Rhizoctonia solani</i> AG-1	KACC 40111	++	++

^aKorean Agricultural Culture Collection (KACC); ^bKorean Collection for Type Cultures (KCTC), *Each value is the mean of inhibition diameter in millimeter from three replicates (± SD); the fungal growth inhibition was reported as (-) between 30 and 45%; (±) between 45 and 60%; (+) between 60 and 80%; and (++) 100%.

signified antibacterial activity (Wang et al., 2001) and the diameter of the inhibition zone was measured after 24 h at 30°C or 37°C depending on optimal temperature requirements for growth of the particular bacterium.

Antifungal activity assays. The *in vitro* antifungal activity of benzoic acid and phenylacetic acid purchased from Sigma-Aldrich Chemical Company was assessed on basis of hyphal radial growth rate of filamentous fungi. The assay was performed by placing an 8-mm diameter plug of growing mycelia onto the center of PDA containing 500 ppm of each compound. Control plates were prepared with 500 ppm of methanol only. The radial growth of mycelia (colony diameter) was measured 5 days after inoculation at 26°C. The percentage of growth inhibition was calculated from the formula used by Chilpa et al. (1997).

$$\% \text{ Inhibition} = (A - B)/A \times 100,$$

where A = mycelia growth in control, and B = mycelia growth in PDA containing active compound.

Destruction of *P. capsici* hyphal morphology by antifungal compounds. The virulent pathogen, *P. capsici* (KACC 40483) was grown on V8 juice media and zoospore suspension was prepared to get a final concentration of 5×10^5 zoospores/ml (Kim et al., 1997). Twenty microliters of fungal spores were placed into plate wells containing 180 μ l of potato dextrose broth and incubated at 30°C in orbital incubator for 24 h. The benzoic acid and phenyl acetic acid were dissolved individually in methanol and added into each well to a final concentration of 250 ppm. Wells containing only potato dextrose broth and methanol were set as control. After 24 h of interaction, the growth inhibition was determined by observing the morphology of hyphae under the microscope (BX41, Olympus, Japan).

Biocontrol of *P. capsici* by *B. cepacia* MPC-7 on pepper *in vivo*. Four weeks old seedlings of pepper (*Capsicum annuum* L.) were transplanted to pots containing 600 g of potting mixture (soil:sand:vermiculite, 2:1:1, vol:vol:vol). Plant growth room was set to 24°C, artificial illumination of 12,000 lux at plant height and 16 h photoperiod. Treatments were CB medium only (M), M plus zoospore inoculation (MP), MPC-7 cultured broth (B) and B plus zoospore inoculation (BP). Treatments were arranged in completely randomized design with three replications. One hundred fifty milliliters of bacterial suspension (3×10^9 colony ml⁻¹) was inoculated three times at 5, 6 and 7 weeks after transplanting (WAT) to B and BP treatments while M and MP were applied with same volume of CB medium only. The zoospore suspension of *P. capsici* (KACC 40483)

previously grown on V8 juice media was prepared to get a final concentration of 5×10^5 zoospores per one milliliter (Kim et al., 1997). At 8 WAT, 50 ml of zoospore suspension was inoculated to the soil after punching four holes to the soil 4 cm from the base of the stem. Only water was applied to control pots in M and B. Destructive samplings were done at 0, 1, 3, 5, 7 and 9 days after pathogen inoculation. Roots were carefully washed and the fresh weight was measured after blot drying. Dry weights of both shoots and roots were measured after drying at 65°C for 48 h. The micro-kjedahl method was used for N determination (Chapman, 1961). The phosphorous concentration of root and shoot was determined by UV spectrophotometer by the method of John et al. (1991). Root mortality was measured using the modified method of Knievel (1973).

Statistical analysis. Experimental data were analyzed using standard analysis of variance (ANOVA) followed by Least Significant Difference tests ($p < 0.05$) using software statistical analytical system (SAS) 9.12 version (SAS Institute Inc, 2008). Standard errors were calculated for all mean values.

Results

Identification of isolated bacteria. Twenty unknown strains showed chitinolytic activities on CM. Among them, one strain having the strongest antifungal activity on dual culture with three fungal pathogens was selected (data not shown). Alignment of 16S rRNA gene sequence through matching with reported 16S rRNA gene sequences using Blast search at Gene Bank Data base of NCBI (Bethesda, MD) showed 100% similarity to *Burkholderia cepacia* strains (GenBank accession number: AF311970, AF097532, AY741337, AB114607, AY946010 and AY946011) and this strain was named as *Burkholderia cepacia* MPC-7.

Phosphate solubilization and organic acid production by MPC-7. MPC-7 solubilized the insoluble phosphate and showed distinct clear zone around the colony in hydroxyapatite agar (HA) media (data not shown). Production of organic acids in culture broth (CB) by MPC-7 was measured by HPLC analysis and it revealed two major peaks which were further identified as gluconic acid and 2-ketogluconic acid. The changes of these organic acid concentrations in the nutrient broth media were monitored as shown in Fig. 1. One day after incubation, the concentrations of gluconic acid and 2-KGA were the same around 1,000 ppm. The concentration of 2-KGA, however, increased continuously to the end of experiment up to 5,580 ppm. On the other hand, the concentration of gluconic acid remained stable around 1,000 ppm.

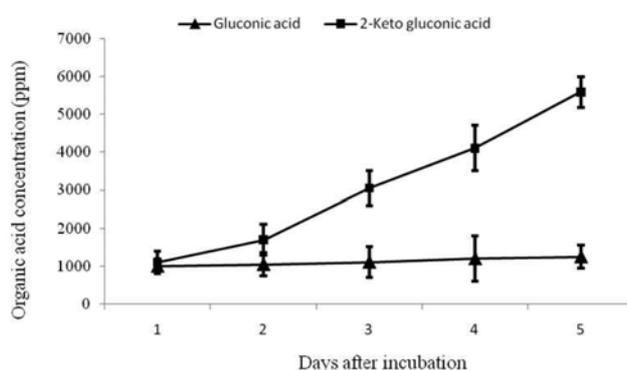


Fig. 1. Changes in concentration of organic acids at chitin medium (CM) containing 0.4% of hydroxyapatite grown with *B. cepacia* MPC-7 at 30°C for 5 days. Mean values were from three replicates (\pm SD).

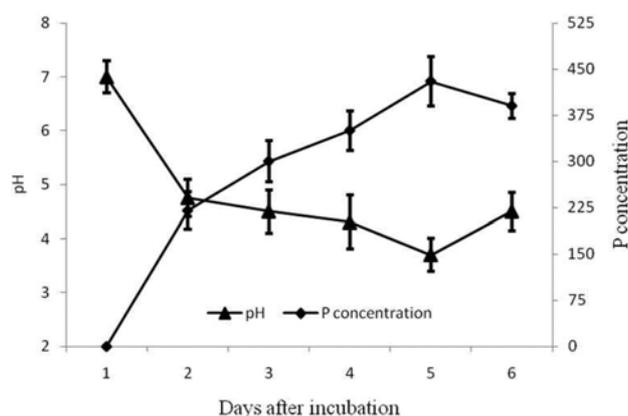


Fig. 2. Changes in pH and P concentrations at chitin medium (CM) containing 0.4% of hydroxyapatite grown with *B. cepacia* MPC-7 at 30°C for 6 days. Mean values were from three replicates (\pm SD).

Measurement of changes in pH and P concentration of the culture media. The pH value had an inverse correlation with the quantity of soluble phosphate. The initial pH of the culture broth set to 7.0 declined sharply to 4.5 (Fig. 2) one day after incubation with MPC-7 and was gradually decreasing thereafter. The maximum concentration of soluble phosphate (430 ppm) was measured after four days of incubation when the pH declined to the minimum (3.7). Similarly, a moderate increase in pH of the culture broth at the last observation correlated to a corresponding decrease in organic acid production.

Extraction, identification and elucidation of antimicrobial compounds. The ethyl acetate-soluble organic fraction (0.4 g) subjected to Sephadex LH-20 column chromatography produced activity at V_e/V_t of 0.86 to 0.92 (102 mg). After purifying this active fraction by ODS column chromatography, an active fraction at 60% MeOH eluate

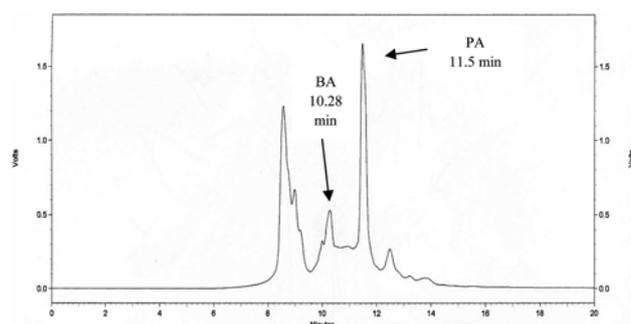


Fig. 3. Typical HPLC chromatogram showing the active peaks of benzoic acid and phenylacetic acid isolated from the culture of *B. cepacia* MPC-7.

(5.4 mg) was obtained. HPLC analysis of the active compound separated into two active peaks with retention times (t_R) of 10.28 min and 11.5 min (Fig. 3). Each compound was identified by GC-EI-MS and determined as benzoic acid (BA) and phenylacetic acid (PA), respectively (Figs. 4A and 4B). They were also confirmed by trimethylsilylation and GC-EI-MS as trimethylsilyl benzoate and trimethylsilyl phenylacetate (data not shown).

Antimicrobial activities of benzoic acid (BA) and phenylacetic acid (PA). Both BA and PA showed distinct clear zones against all types of bacteria tested in this experiment and the inhibition diameter ranged from 11.0 to 17.3mm at 500 ppm (Table 1). The strongest antibacterial activities of BA and PA in term of wider clearance zones (17.3 and 16.2, respectively) were observed against *X. campestris* pv. *vesicatoria*. Similarly, the growth inhibition of *S. cerevisiae* (yeast) was also observed for having the wider clear zones around the paper discs impregnated with BA and PA.

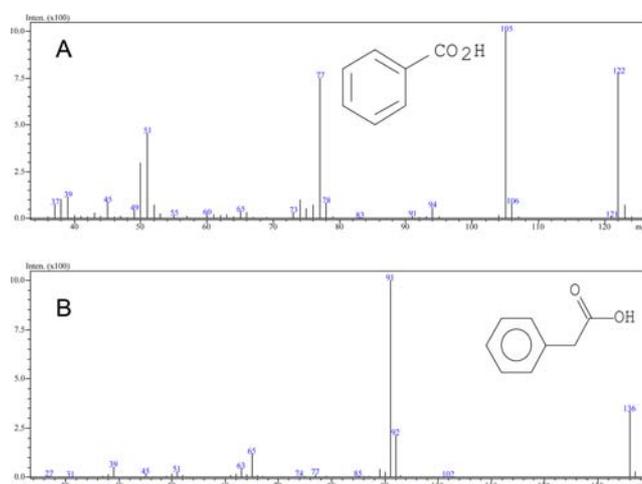


Fig. 4. GC-EI-MS spectra and structures of benzoic acid (A) and phenylacetic acid (B) isolated from the culture of *B. cepacia* MPC-7.



Fig. 5. Hyphal morphology of *P. capsici* grown in potato dextrose broth treated with 250 ppm of methanol (A), benzoic acid (B) and phenylacetic acid (C) after incubation for 24 h at 30°C

Similar to the antibacterial activity, both BA and PA consistently showed a broad spectrum of antifungal activities. Complete inhibitions to the growth of *B. cinerea*, *D. bryoniae*, *F. oxysporum* f. sp *gladioli*, *P. capsici*, *P. aphanidermatum* and *R. solani* AG-1 were observed in media containing 500 ppm benzoic acid. Furthermore, PA had perfect growth inhibition over *P. capsici*, *P. aphanidermatum* and *R. solani* AG-1. However, the *A. brassicicola* seemed to be relatively tolerant to both compounds and showed a moderate level of growth inhibition (between 45 and 60%). *F. oxysporum* f. sp *melonis* was less affected at 500 ppm of phenylacetic acid and the lowest level of growth inhibition (< 45%) was recorded.

Destruction of *P. capsici* hyphal morphology by anti-fungal compounds. Both 250 ppm of BA and PA affected the normal growth of *P. capsici*. Through microscopic studies, the deformation of fungal hyphae treated with both compounds was quite distinct from the normal one after incubation for 24 h at 30°C (Fig. 5). Moreover, the architecture and angle of hyphal branching were also severely altered. The reaction of BA and PA to the fungal cell wall caused the hyphae to be severely distorted, deformed and somewhat enlarged at the tip while the normal shape and

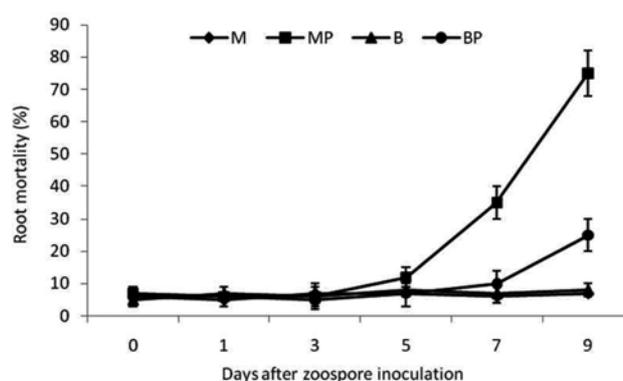


Fig. 6. Changes of root mortality in pepper as influenced by culture broth medium only (M), M + *P. capsici* (MP), MPC-7 cultured broth (B) and B + *P. capsici* (BP). Mean values are from 3 replicates. Mean values were from three replicates (\pm SD).

branching was maintained in control (Fig. 5C).

Biocontrol of *P. capsici* by *B. cepacia* MPC-7 on pepper *in vivo*. The root mortality of pepper plants did not vary among treatments during the earlier days after the inoculation of *Phytophthora* zoospores. Notably at fifth day, the pathogenic attack of zoospore was first conspicuous due to

Table 2. Root and shoot characteristics, N and P concentrations of pepper plants at 9 days after inoculation of *P. capsici* zoospore suspension

Treatment	Shoot			Root			Plant Height (cm)	Leaf Area (cm ²)
	Dry Weight (g)	N Conc (%)	P Conc (%)	Dry Weight (g)	N Conc (%)	P Conc (%)		
M	1.89 \pm 0.2b	2.33 \pm 0.4b	1.75 \pm 0.1a	0.16 \pm 0.02a	1.64 \pm 0.2a	1.75 \pm 0.1b	37.56 \pm 2.5c	716.73 \pm 32b
MP	1.37 \pm 0.1c	2.36 \pm 0.3b	1.71 \pm 0.1a	0.14 \pm 0.02a	1.74 \pm 0.1a	1.53 \pm 0.2c	36.43 \pm 2.8c	476.47 \pm 24c
B	2.21 \pm 0.2a	2.6 \pm 0.2a	1.87 \pm 0.2a	0.20 \pm 0.01a	1.77 \pm 0.1a	1.92 \pm 0.3a	43.28 \pm 3.2a	778.08 \pm 28a
BP	2.07 \pm 0.2 ab	2.4 \pm 0.3b	1.8 \pm 0.1a	0.18 \pm 0.01a	1.78 \pm 0.1a	1.87 \pm 0.2ab	40.08 \pm 3.1b	734.71 \pm 33b

M, culture broth medium only; MP, M + *P. capsici*; B, MPC-7 cultured broth; BP, B + *P. capsici*; each value is mean of three pepper plants from three replicates, different letters within each column ($F > 4$, $df = 3, 8$, $P < 0.05$) are significantly different according to LSD test.

a sudden increase of root mortality in MP. The increase was very rapid at day 7 and 9 while the control plants in M and B did not show any increase in root mortality (Fig. 6). In spite of zoospore inoculation, the root death of plants in treatment BP was nullified and the root mortality was reduced as much as 50% compared to that of MP. Consequently, plants in treatment MP wilted as early as five days after zoospore inoculation while those in BP showed no wilting. The plants in BP had similar growth performance with those in M which did not receive any zoospore inoculation. On the other hand, plants in B had the greatest leaf area, higher dry weight; shoot N and root P concentrations and greater leaf area than those in M and MP treatments (Table 2). The plants in BP also had higher root P, shoot dry weight and greater leaf area than those in MP although both received *P. capsici* zoospore suspension. At the end of the experiment, the plants in B and BP were taller than the plants in M and MP.

Discussion

The hydroxyapatite agar media has been previously used to screen the phosphate solubilization bacteria for the appearance of clear zone on the surface of media (Kim et al., 1997a; Kim et al., 1997b). Accordingly, *B. cepacia* MPC-7 initially selected as strongest chitinolytic bacterial strain was considered as phosphobacteria because it also solubilized the insoluble phosphate present in the hydroxyapatite agar media. Generally, the mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids (Kim et al., 1997a). HPLC chromatogram showed two major peaks which were identified as gluconic acid and 2-Keto gluconic acid having similar retention times with those of our previous reports (Na et al., 2009; Park et al., 2005). Quantitative analysis showed that the concentration of soluble P increased with decrease in pH of culture medium (Fig. 2). This decrease in pH was related to the production of gluconic acid and 2-KGA (Fig. 1). The concentration of 2-KGA increased gradually with incubation time and it seemed to be further oxidization of gluconic acid to 2-KGA. Similar trend of changes in 2-KGA was also found in our previous report (Hwangbo et al., 2003) in which *Enterobacter intermedius* possessed a strong ability to solubilize insoluble phosphate, and oxidized glucose to gluconic acid and sequentially to 2-KGA. It is also in agreement with Ta et al. (2006) who reported the production of gluconic acid and 2-KGA by *B. cepacia* strain caused the solubilization of tricalcium phosphate. The mechanism of P solubilization has been previously reported by Rodriguez and Fraga (1999) as gluconic acid diffuses freely outside of the cells and might release high amounts

of soluble P from insoluble P, by supplying both protons and cation complexing organic acid anions. These organic acids may reduce pH and act as chelating agents, forming complexes with Ca, Fe, or Al, and thereby releasing the phosphates to solution (Viruel et al., 2011). Finally, the insoluble form of phosphorous is converted into soluble monobasic (H_2PO_4) and dibasic (HPO_4^{2-}) ions, a process referred to as mineral phosphate solubilization. This leads to an increase in the availability of phosphorous to plants and in turn the plant uptake (Gyaneshwar et al., 2002). Notably, the maximum solubilization of P observed at 4th day of incubation was coincided with the maximum pH decline of culture broth (Fig. 3). This result was further strengthened by Chen et al. (2006) who reported that bacterial strains such as *Arthrobacter* sp. and *Serratia marcescens* which synthesized maximum number of organic acids showed maximum decline in pH 4.9 with higher levels of P solubilization.

The MPC-7 produced benzoic acid in CB media. Rarely, one report about production of BA in minimum mineral medium by *B. cepacia* was available (Philippe et al., 2001). The halo zone test and radial growth inhibition tests, 500 ppm of BA showed very strong antimicrobial activity against yeast, all bacterial and most fungal pathogens tested. The mechanism of inhibition to the growth of *Saccharomyces cerevisiae* was previously reported by Krebs et al. (1983) as the benzoic acid inhibited the growth by lowering the intracellular pH, which inhibited glycolysis, especially by inhibition of phosphofructokinase. Although the mechanism of its inhibition to bacteria was not known yet, lipophilic acid such as BA is known to inhibit the active uptake of some amino and oxoacids in *E. coli* and *B. subtilis* (Russell and Chopra, 1996).

PA was isolated from other micro-organisms such as *Pseudomonas* sp. (Jae et al., 1999), *Streptomyces humidus* (Hwang et al., 2001) and *Bacillus licheniformis* (Kim et al., 2004). However, this is the first report about production of PA by *B. cepacia*. In this study, 500 ppm PA consistently showed strong antimicrobial activity against all bacteria, and a varying level of inhibition against all fungal pathogens tested (Table 1). The mode of action of phenyl acetic acid on all stages of the life cycle of *P. capsici*, including zoospore motility, zoospore germination, and mycelia growth may be connected with impairment of the energy generation system (Lee et al., 2004). Our finding is in agreement with Kim et al. (2004) who reported that the BA and PA produced by *Bacillus licheniformis* showed strong antimicrobial properties against several bacteria, fungi and yeast.

By microscopic observation, the fungal cell walls of *P. capsici* were severely affected by 250 pm of BA and PA. Each compound might be presumably associated with biochemical changes leading to structural modification of

P. capsici cell walls. As a consequence, the normal morphology of hyphae was distinctly destroyed and the architecture of branching pattern was severely disturbed resulting to abnormality of hyphae (Fig. 5).

The biocontrol potential of MPC-7 was demonstrated in pot trial. As shown in Fig. 6, the root mortality of plants treated with MPC-7 was significantly lower than the controls. The result demonstrated that the drenching of rhizosphere by MPC-7 and earlier root colonization prevented the invasive zoospore infection of the soil borne pathogen *P. capsici*. The production of antimicrobial compounds such as benzoic acid and phenyl acetic acid by MPC-7 might play a key role of disease protection by inhibiting the growth of fungal hyphae (Fig. 5). Moreover, these antibiotics might individually or synergistically interact to prevent zoospore germination or directly destroy them. Therefore, the hyphal growth was supposed to be prevented by these antibiotics from the beginning of the pathogen lifecycle. This finding was supported by Lee et al. (2004) who reported that the concentration of 10 µgml⁻¹ of phenylacetic acid was sufficient to lyse most of *P. capsici* zoospores. In our current study, growth of pepper plants treated with bacterial cultures was significantly enhanced and resulted in excellent protection from disease. This elegant result demonstrated that drenching of root zone with antagonistic bacteria would be a very practical method and it can be efficiently applied in field through drip irrigation facilities.

As shown in Table 2, the highest P concentration in roots treated with MPC-7 pinpointed the potential of this strain to enhance available P. The subsequent higher uptake of P by those plants might contribute better growth through greater photoperception and photosynthetic efficiency that finally increased the dry matter assimilation, shoot N concentration and plant height (Table 2). Moreover, the significance of greater leaf area, dry weight and height of plants treated with MPC-7 may also be supplemented by the production of phenylacetic acid which has been known as a growth- and development-promoting compound in maize (Sarwar and Frankenberger, 1995) and a natural auxin in the shoots of higher plants, such as barley, corn, tobacco and tomato (Wightman and Lighty, 1982). In conclusion, the present study demonstrated that *B. cepacia* MPC-7 is considered as a candidate for the biological fertilizer as well as antimicrobial agent for pepper plants.

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