INTRODUCTION

Many of the chemical fungicides currently used for plant disease control are classified as carcinogens by the Environmental Protection Agency (EPA). They are toxic to wildlife and other non-target species. Biological control offers an attractive alternative to synthetic chemical fungicides. Biopesticides that include living organisms and their naturally produced compounds can be safer, more biodegradable, and less expensive to develop. Various microorganisms in soil, including Enterobacter aerogenes, Pseudomonas fluorescens, Pseudomonas cepacia, and Bacillus species, have been reported for their role in promotion of plant growth and biological control of plant pathogenic microorganisms. It is believed that plant growth-promoting rhizobacteria act by displacing or antagonizing plant pathogenic microorganisms. Many of these rhizobacteria produce antibiotics, the production of which is strongly correlated with inhibition of various pathogenic microorganisms in vitro and disease suppression in vivo.

The use of bacteria like Pseudomonas sp. and Bacillus sp., have been investigated because of their properties to produce antifungal metabolites and protect plants from fungal infection (Radheshyam et al. 1990; Moita et al. 2005; Siddiqui et al. 2005; Nourozian et al. 2006). Bacteria of the genus Bacillus produce a variety of peptide antibiotics that are antibacterial and/or antifungal. Several Bacillus species, including B. subtilis, B. pumilus and B. cereus, have been shown to be antagonistic to plant pathogenic fungi and bacteria. Phytopathogen-antagonistic strains of B. subtilis have been reported to produce two peptide antibiotics: bacilysin, a dipeptide that inhibits yeast and bacteria; and fengycin (fengymycin), a lipopeptide antagonistic against phytopath-
ogenic fungi such as *Rhizoctonia solani* (Loeffler et al. 1986; Vanittanakom et al. 1986).

Stabb *et al.* (1994) have identified certain strains belong to *B. subtilis*, *B. cereus*, *B. mycoides*, and *B. thuringiensis* exhibit antifungal activity. These strains have been shown to produce zwittermicin-A and/or kanosamine (Milner *et al.* 1996) that are effective against the soil borne disease damping off, caused by *Phytophthora medicaginis*, *P. nicotianae*, *P. aphanidermatum* or *Sclerotinia minor*. Zwittermicin-A is a water soluble, acid stable linear aminopolyol molecule (He *et al.* 1994) with broad spectrum activity against many fungal and bacterial plant pathogens. The application of spore-based formulations of certain *B. cereus* strains to soybean seeds or the soil surrounding the seeds has been shown to improve soybean yield at field sites (Osburne *et al.* 1995).

Kanosamine also inhibits a broad range of fungal plant pathogens and a few bacterial species (Milner *et al.* 1996). *Bacillus licheniformis* strain PR1-36a, isolated from a rhizosphere of perennial ryegrass (*Lolium perenne* L.) exhibited strong antagonism against filamentous fungi such as *Rhizoctonia solani* and *Magnaporthe poae*. It produces a diffusible antifungal principle that can be obtained by acid precipitation of the culture filtrate followed by ethanol extraction.

Loeffler *et al.* (1986) reported that *B. subtilis*, *B. pumilus*, *B. licheniformis*, and *B. coagulans* strains produced various antibiotics with antifungal and antibacterial activity. Extracellular antifungal metabolites produced by *Bacillus pumilus* inhibited mycelial growth of many species of *Aspergillus*, *Penicillium* and *Fusarium* (Munimbazi and Bullerman 1998). *B. pumilus* produced bacilysin and iturin A. Bacilysin is a very small compound with a molecular weight of 270 that inhibits only yeast. The iturins, which are soluble in polar solvents, have broad antifungal and antibacterial activity. Antifungal activity of *Bacillus licheniformis* strains were enhanced through mutation and transformation (Cornea *et al.* 2003; Mateescu *et al.* 2004). The present study is aimed to isolate the bacterial strains with antifungal property and to establish their biocontrol potentials.

**MATERIALS AND METHODS**

1. **Bacterial and fungal isolates**

Soil samples were collected from bean rhizosphere and dry fields in Ipyeong-ri, Jeollanam-do province, South Korea. Samples were serially diluted and plated on nutrient agar
(NA) plates. The colonies were bio-assayed on potato dextrose agar (PDA) plates against *Rhizoctonia solani* and *Alternaria alternata*. Agar discs from the actively growing region of fungal pathogens were collected and placed at the center of the PDA plates. Bacterial isolates were streak inoculated on the same plate at 2.5 cm away from the fungal disc and incubated at 28±2°C for 3~5 days. The plates were observed for the presence of a fungal growth inhibition zone. The isolates showing antifungal property by inhibiting the fungal growth were selected and maintained as 25% glycerol stock in nutrient broth under −80°C as well as refrigerated conditions. Fungal phytopathogens used in bioassay as listed in Table 1, were maintained in PDA plates and stored under refrigerated condition.

### 2. Phylogenetic relationship of antifungal metabolite producing bacterial isolates

Bacterial isolates with antifungal trait were identified based on 16S rDNA sequence analysis. Genomic DNA was extracted by using Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA). 16S rDNA was amplified by using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991). The sequences were determined by fluorescent dye terminators method using ABI prism™ Bigdye™ terminator cycle sequencing ready reaction kit V.3.1. Products were run on ABI 3730XL capillary DNA sequencer (ABI prism 310 Genetic analyzer, Tokyo, Japan). Sequences were subjected to BLASTn analysis with the NCBI data base and phylogenetic trees were generated with NJPLOT programme of CLUSTAL X (Thompson et al. 1997).

### 3. Extraction of antifungal metabolite from *Bacillus* isolates

*Bacillus* isolates with antifungal activity were grown in 20 ml of potato dextrose broth for 62 h, 200 rpm at 28±2°C.
The culture supernatant was prepared by centrifugation at 5,000 rpm for 20 min and added with an equal volume of n-hexane. The organic phase was separated with separating funnel and tested for antifungal activity with *A. alternata*. The same aqueous phase was again extracted with equal volume of chloroform, ethyl acetate and n-butanol sequentially. Aqueous as well as organic fractions were tested for antifungal activity by disc diffusion assay with *A. alternata*. Active crude extracts were dried completely and dissolved in HPLC-grade methanol.

4. Chitinase activity and mineral phosphate solubilization

Ability of *Bacillus* isolates to produce chitinase was tested on nutrient agar medium supplemented with 0.5% colloidal chitin. Colloidal chitin was prepared by the method of Rodríguez-Kabana *et al.* (1983) by partial hydrolysis of chitin (Sigma, St. Louis, USA) with 10 N HCl for 2 h at room temperature. The colloidal chitin was washed several times with large volumes of distilled water to adjust the pH to 7.0. Phosphate solubilizing ability was detected by spotting the isolates on Pikovskaya agar (Pikovskaya 1948) as well as NBRIP-BPB agar plates (Mehta and Nautiyal 2001). Plates were then incubated at 28°C for 3 days and observed for the clearing zone around the colonies.

5. Radiation sensitivity

Cell pellets were collected by centrifuging 20 ml of a log phase bacterial isolate cultured in the LB broth. Pellets were washed twice with sterile-distilled water and suspended (~10^7 ~ 10^8 CFU ml^-1). 500μl of cell suspensions were transferred to 1.5 ml micro-centrifuge tubes and irradiated at different doses expressed in kGy, as shown in Fig. 3. Samples were irradiated in a cobalt-60 irradiator (capacity: 250000 Ci, dose rate 920 Gy/hr, AECL) at the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, South Korea. The bacterial population in irradiated samples was determined by serial dilution and plate count method. The D_{10} value was determined by plotting a radiation dose (kGy) on the X-axis and a survival ratio on the Y-
axis. The negative reciprocal of the slope indicated the \( D_{10} \) value. \( LD_{99} \) value, the radiation dose required to kill 99% of viable bacterial cells was determined by using the formula \( LD_{99} = \log (0.01) \text{/slope} \).

6. Scanning electron microscopy

Bacterial cells were fixed directly in glutaraldehyde buffer for 4 h and then dehydrated. Mycelium of \( R. solani \) was treated with a crude antifungal metabolite with a concentration of 10 mg ml\(^{-1} \) of water. Hyphae were removed after 5 h of incubation, fixed in glutaraldehyde buffer for 4 h in room temperature, dehydrated in graded alcohol, and dried. Gold sputter-coating was carried out under reduced pressure in an inert argon gas atmosphere (Agar sputter coater P7340). After sputter-coating, specimens were examined under a scanning electron microscope (JSM 6390) operated at 30 kV.

RESULTS AND DISCUSSION

Rhizobacteria that are able to colonise the root region take advantage of the root exudates released by the plant. This interaction is beneficial both for the plant as well as the bacteria. The presence of non-pathogenic bacteria at the roots confers protection against pathogenic microorganisms. This protection by bacteria can be mediated due to production of antibiotics harmful to other microorganisms (Wulff et al. 2002; Bais et al. 2004). Formation of biofilm on plant roots by the bacteria can make the plants less sensitive to infection (Bais et al. 2004; Rudrappa et al. 2008). In this study, bean rhizosphere soil and dry field soil samples were serially diluted and plated on nutrient agar plates. Colony with different morphology and colour were purified and stored under refrigerated condition. Bacterial isolates were tested for their antifungal activity against \( A. alternata \) by dual culture assay. Four bacterial isolates that inhibited the growth of \( A. alternata \) were selected and designated as A1, A2, A3 and A4. The antagonistic activity of these isolates against other fungal pathogens as listed in Table 1 was demonstrated by dual culture assay. All the \( Bacillus \) isolates inhibited the growth of \( A. alternata, A. solani, Botrytis cinerea, \) and \( Rhizoctonia solani in vitro \) by secreting anti-

![Fig. 4. Effect of crude antifungal metabolite(s) on \( A. alternata \). A) Untreated hyphae, B) Hyphae treated with crude antifungal metabolite(s) showing depressions (B-1), shriveled and empty hyphae (B-2) and bursting and hyphal lysis (B-3).](image)
fungal compound(s) out of the bacterial cells, as indicated by the formation of inhibition zone. These isolates did not produce inhibition zone against other phytopathogenic fungi including *Cladosporium*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Phytophthora capsici*, *Pythium ultimum* and *Sclerotinia sclerotiorum* though the fungal growth was reduced. Three days after incubation in dual culture assay, the fungi overgrew the bacterial colony.

All the four bacterial isolates are Gram positive rods. Cell size ranges from 0.3\(\mu\)m ~ 0.4\(\mu\)m width and 1.3\(\mu\)m ~ 1.8\(\mu\)m length. 16S rDNA analysis indicated that the isolates belong to Genus *Bacillus*. To study the phylogenetic relationship, neighbor-joining dendrogram was generated for our isolates and representative *Bacillus* sequences from GenBank. Isolate A1 clustered with *Bacillus licheniformis* while other isolates A2, A3 and A4 clustered together with *B. pumilus* (Fig. 1). We attempted to extract the antifungal metabolites of our isolates. None of the n-hexane extracts showed the antifungal activity against *A. alternata*. Chloroform extract of isolate A2 partially inhibited the fungal growth. Chloroform extracts of other isolates did not inhibit the test fungus. Most of the antifungal metabolites from these isolates were extracted with n-butanol and showed strong zone of inhibition. Ethyl acetate extract of isolates A1, A3 and A4 partially inhibited the fungus and longer incubation time allowed the fungus to overgrow the antibiotic discs. The *Bacillus* isolates were also tested for the production of chitinase and mineral phosphate solubilization. Except isolate A4, others produced chitinase enzyme. None of our isolates solubilized mineral phosphate in Pikovskaya agar medium. The results were also confirmed in NBRIP-BPB agar plates. Radiation mutagenesis is considered as a tool to modify the traits of rhizobacteria in order to enhance their activity and to understand their basic mechanisms of plant growth promotion. We initially studied the radiation sensitivity of our *Bacillus* isolates. The D\(_{10}\) and LD\(_{99}\) values were determined by plotting a radiation dose (kGy) on the X-axis and a survival ratio on the Y-axis. Isolates A1, A2, A3 and A4 recorded the D\(_{10}\) values of 0.25, 3.35, 5.80, and 0.76 kGy respectively. LD\(_{99}\) values of isolates A1, A2, A3 and A4 are 0.50, 6.69, 11.60, 1.53 kGy, respectively. Isolate A3 is comparatively resistant to radiation while isolate A1 is highly susceptible. *Bacillus* isolates were exposed to gamma radiation at their LD\(_{99}\) dose. Mutant clones were stored as 25% glycerol stocks under -80\(^\circ\)C. Mutant clones can be screened either for enhanced antifungal activity or defective mutants. Several antifungal antibiotics caused morphological abnormalities on filamentous test fungi. Polyoxyin caused formation of characteristic bulges or swellings on the growing hyphae and spores of sensitive filamentous fungi such as *Piricularia oryzae* and *Mucor racemosus*. Tunicamycin, a specific inhibitor of glycoprotein synthesis in yeast, caused bursting, swelling of *Penicillium chrysogenum* (Yoshiki et al. 1976). Griseofulvin induced hyphal curling of several fungi tested. The most remarkable curling was observed with *Paecilomyces variotii*. Inhibitors of protein synthesis such as cycloheximide and blasticidin S and inhibitors of energy metabolism such as antimycin and oligomycin showed slight or no effect on the morphology of the tested organisms in the case of higher concentrations than their MIC values (Gunji et al. 1983). Microscopic observation on the effect of crude metabolites of our Bacillus isolates on *A. alternata* hyphal morphology was performed under scanning electron microscope. Crude metabolite caused drastic changes on

### Table 1. Antifungal activity of *Bacillus* strains against phyto-pathogens

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<thead>
<tr>
<th>Fungus</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
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<tr>
<td><em>Alternaria alternata</em></td>
<td>7.25 ± 1.3</td>
<td>6.75 ± 1.0</td>
<td>8.50 ± 0.6</td>
<td>8.25 ± 0.6</td>
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<tr>
<td><em>Alternaria solani</em></td>
<td>5.75 ± 0.5</td>
<td>4.50 ± 0.6</td>
<td>6.25 ± 1.0</td>
<td>7.00 ± 0.8</td>
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<td><em>Botrytis cinerea</em></td>
<td>4.75 ± 0.5</td>
<td>4.25 ± 1.0</td>
<td>7.00 ± 1.4</td>
<td>6.75 ± 1.0</td>
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<tr>
<td><em>Cladosporium sp.</em></td>
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<td><em>Colletotrichum gloeosporioides</em></td>
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<td><em>Fusarium oxysporum</em></td>
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<td><em>Phytophthora capsici</em></td>
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<td><em>Pythium ultimum</em></td>
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<tr>
<td><em>Rhizoctonia solani</em></td>
<td>4.50 ± 0.6</td>
<td>6.00 ± 0.8</td>
<td>6.25 ± 0.5</td>
<td>4.75 ± 0.5</td>
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<tr>
<td><em>Sclerotinia sclerotiorum</em></td>
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hyphal morphology. Depressions were developed on the surface of treated hyphae. Appearance of shrunken and collapsed hyphae could be due to the leak of cell wall or changes in membrane permeability. Hyphal swelling and lysis were also observed. In conclusion, present work established the antagonistic activity of *Bacillus* isolates A1, A2, A3 and A4 against major phytopathogens. Further research on purification and characterization of antifungal metabolites will help to understand the detailed mechanism of plant disease suppression through biological control.

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REFERENCES


