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Isolation and *In vitro* and *In vivo* Antifungal Activity of Phenylacetic acid Produced by *Micromonospora aurantiaca* Strain JK-1

Hyo Jin Kim, In Sun Hwang, Beom Seok Kim and Byung Kook Hwang*

Division of Bioscience and Technology, College of Life and Environmental Sciences, Korea University, Seoul 136-713, Korea

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The actinomycete strain JK-1 that showed strong inhibitory activity against some plant pathogenic fungi and oomycetes was isolated from Jung-bal Mountain in Ko-yang, Korea. The strain JK-1 produced spores singly borne on sporophores and the spores were spherical and 0.9-1.2 μm in diameter. The cell wall of the strain JK-1 contained *meso*-diaminopimelic acid. The actinomycete strain JK-1 was identified as the genus *Micromonospora* based on the morphological, physiological, biochemical and chemotaxonomic characteristics. From the 16S rDNA analysis, the strain JK-1 was assigned to *M. aurantiaca*. The antibiotic MA-1 was purified from the culture broth of *M. aurantiaca* JK-1 using various purification procedures, such as Diaion HP20 chromatography, C18 flash column chromatography, silica gel flash column chromatography and Sephadex LH-20 column chromatography. ^{1}H-, ^{13}C-NMR and EI mass spectral analysis of the antibiotic MA-1 revealed that the antibiotic MA-1 is identical to phenylacetic acid. Phenylacetic acid showed *in vitro* inhibitory effects against fungal and oomycete pathogens *Alternaria mali, Botrytis cinerea, Magnaporthe grisea, Phytophthora capsici* and yeast *Saccharomyces cerevisiae* at < 100 μg ml^{-1}. In addition, phenylacetic acid completely inhibited the growth of *Sclerotinia sclerotiorum, Bacillus subtilis, Candida albicans, Xanthomonas campestris* pv. *vesicatoria* at < 500 μg ml^{-1}. Phenylacetic acid strongly inhibited conidial germination and hyphal growth of *M. grisea* and *C. orbiculare*. Phenylacetic acid showed significantly high levels of inhibitory effect against rice blast and cucumber anthracnose diseases at 250 μg ml^{-1}. The control efficacies of phenylacetic acid against the two diseases were similar to those of commercial compounds tricyclazole, iprobenfos and chlorothalonil in the greenhouse.

Keywords : antifungal activity, *Micromonospora aurantiaca*, phenylacetic acid

In an effort to isolate novel natural products, the use of rare actinomycete strains other than *Streptomyces* spp. has steadily increased (Tanaka and Omura, 1993). Recently, actinomycetes which belong to the genera *Micromonospora* and *Microbispora* have been of interest as a new source of industrially useful metabolites, such as antibiotics and degraders of natural rubber (Jendrossek et al., 1997). Recently, the genus *Micromonospora* has attracted interest as a novel source of antibiotics. Many of aminoglycoside type (gentamicins, sisomicin, G-418, verdamicin, sagamicin, and G-52), the macrolide type (megalomicins, rosamicin, juvenimicin, and M-4365) and miscellaneous types (iodinin, tetrenolin, and bottromycins) (Wagman and Weinstein, 1980) have been found in strains of genus *Micromonospora*. More recently, novel therapeutic compounds, such as megalomicin and calicheamicin have been shown to have anticancer, antiviral, and antiparastic activities (Alarcon et al., 1988).

The genus *Micromonospora* was defined on a chemotaxonomic and phylogenetic basis (Kawamoto, 1989; Koch et al., 1996; Kroppenstedt, 1985; Lee et al., 1999), as well as on morphological properties. Reclassification of *Micromonospora* species was carried out by Kasai et al., (2000) on the basis of *gyrB* gene sequence analyses and DNA-DNA hybridization experiments. Consequently, the genus *Micromonospora* is considered to accommodate 15 species: *M. aurantiaca, M. carbonacea, M. chalcea, M. chersina, M. coerulea, M. echinospora, M. gallica, M. halophytica, M. inositola, M. matsumotoense, M. nigra, M. olivasterospora, M. pallida, M. purpureochromogenes* and *M. rosaria* (Wagman and Weinstein, 1980).

Welsch (1942) reported first the antibiotic activity from *Micromonospora*. After that, the aminoglycoside antibacterial antibiotic gentamicin was isolated from two species, *M. purpurea* NRRL 2953 and *M. echinospora* NRRL 2985, and two subspecies, *M. echinospora* subsp. *ferruginea* NRRL 2995 and subsp. *pallida* NRRL 2996 (Weinstein, 1964). Various aminoglycoside type antibiotics such as sisomicin (*M. inyoensis* NRRL 3292), verdamicin (*M. grisea* NRRL 3800), antibiotic G-52 (*M. zionensis* NRRL 5466), antibiotic G-418 (*M. rhodorangea* NRRL 5326), antibiotic JI-20 (*M. purpurea* NRRL 2953), and fortimicins (*M. olivoasterospora*), antibiotic 460 (*M. chalcea* var. *flavida* NRRL 3222), and sagamicin (*M.*

*Corresponding author.
Phone) +82-2-3290-3061, FAX) +82-2-925-1970
E-mail) bkhwang@korea.ac.kr

sagamiensis ATCC21803) were discovered from the *Micromonospora* species. Neomycin B is the first example of an important aminoglycoside produced by a *Streptomyces* species and by a *Micromonospora* species (Wagman, 1973). The macrolide antibacterial antibiotics from *Micromonospora* species include megalomicins (*M. megalomicea* var. *megalomicea* NRRL 3274 and var. *nigra* NRRL3275), rosaramicin (*M. rosaria* NRRL 3718), juvenimicins (*M. chalcea* var. *izumensis* ATCC21561), M-4365 complex (*M.capillata* sp. nov.) and erythromycin B (*Micromonospora* sp.) (Wagman, 1980). The halomicins were isolated from two previously undescribed species of *Micromonospora, M. halophytica* NRRL 2998 and *M. halophytica* var. *nigra* NRRL 3097 (Weinstein, 1968). The rifamycin complex was produced by *M. ellipsospora* NRRL 8021. Moreover, miscellaneous antibiotics, such as iodinin (Gerber and Lechevalier, 1964) and tetrenolin (Pagani et al., 1973) have been found in the various species of *Micromonospora.*

The various species of *Micromonospora* were identified as producers of antifungal substances. The neihumicin was isolated from the culture broth of a soil isolate identified as *Micromonospora neihuensis* (Wu et al., 1988). Neihumicin showed *in vitro* antifungal activity against *Saccharomyces cerevisiae* ATCC 9763. Cooper et al. (1987) have discovered a novel antifungal compound, Sch 37137, produced by a *Micomonospora* sp. The compound was active against species of *Candida* and *Dermatophytes* sp. (Cooper et al., 1987). This compound is closely related to A 19009 (Shirling, 1966) previously isolated from a *Streptomyces* sp., where an epoxide replaces a double bond in the molecule. *Micromonospora* sp. M39 was identified as a producer of antifungal substances, which were inhibitory to rice blast pathogen *Pyricularia grisea* MPO 293 (Ismet et al., 2004). A combination of starch, glucose and glycerol at 1% concentration as nitrogen source in basal medium enhanced the antifungal activity of the strain M39. In addition, *Micromonospora* sp. M39 produced a number of substances, such as 2,3-dihydroxybenzoic acid, phenylacetic acid and cervinomycins (Ismet et al., 2004). The galbonolides A and B were recently isolated from a *Micromonospora* sp. (Shafiee, 2001). The 21-hydroxy compounds prepared by biotransformation of the galbonolide A and B exhibited significant antifungal activity against a number of pathogenic fungi including *Candida* sp. and *Cryptococcus neoformans* (Shafiee, 2001). Streptimidone was isolated from *M. coerulea* strain A058 (Kim et al., 1999). Streptimidone completely inhibited the growth of plant pathogenic fungi, such as *Phytophthora capsici, Magnaporthe grisea, Didymella bryoniae* and *Botrytis cinerea.* Two antifungal antibiotics, spartanamicins A and B, also were produced by a culture of *Micromonospora* spp. strain no. MSU-43097 (ATCC 53809) (Kinoshita, 1991). Spartanamicins B had antifungal and antibacterial activity against *Candida albicans, Aspergillus, Cladosporium, Cryptococcus, Rhodotorula* and *Staphylococcus* spp. This compound related to anthracycline antibiotics with marginal antifungal activity may have different configurations in their sugar moieties, thus exhibiting the difference in their solubilities and antifungal activities (Kinoshita, 1991). The macrolide antibiotic M-4365 produced by *Micromonospora* showed antibacterial activity against gram-positive and gram-negative bacteria, such as *Staphylococcus aureus, Salmonella typhimurium* and *Pseudomonas aeruginosa* A_3. (Tamotsu, 1977; Toutaro, 1978). The mycinamicins are macrolide antibiotics produced by *Micromonospora griseorubida* FERM BP-705, which have strong antibacterial activity against gram-positive bacteria (Satoi, 1980).

In the search for microorganisms producing antifungal antibiotics useful for plant disease control, we isolated actinomycete strain JK-1 from Jung-bal Mountain in Koyang, Korea (2003). The actinomycete strain JK-1 was identified as *M. aurantiaca.* The antibiotic MA-1 was purified from the culture broth of *M. aurantiaca* using various purification procedures. Structure of the antibiotic substance MA-1 was elucidated by spectral analyses. *In vitro* antifungal activity of the antibiotic MA-1 was evaluated against various microorganisms. *In vivo* control efficacy of the antibiotic MA-1 against rice blast and cucumber anthracnose diseases was evaluated under the greenhouse conditions by comparing with commercial fungicides tricyclazole, iprobenfos and chlorothalonil. This paper reports the isolation process for the antibiotic MA-1 from *M. aurantiaca* strain JK-1, its molecular characteristics and the *in vitro* and *in vivo* antifungal activity.

Materials and Methods

Isolation of antifungal actinomycete strain JK-1 from soil. A soil sample was collected from Mt. Jung-bal, Koyang, Korea. Soil samples were taken from 10-20 cm deep from the soil surface and air-dried at room temperature for 10 days. Dried soil samples (5 g) were mixed with 50 ml of sterile water in a 250 ml Erlenmeyer flask. These flasks were shaken at 120 rpm for 30 min. The soil mix was diluted to be suspended to 10^{-3} and 10^{-4}. The diluted samples (0.2 ml) were plated on the humic acid vitamin agar (1 g humic acid, 0.5 g Na_2HPO_4, 1.71 g KCl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 0.5 mg thiamine-HCl, 0.5 mg riboflavin, 0.5 mg niacin, 0.5 mg pyridoxin-HCl, 0.5 mg inositol, 0.5 mg Ca-pantothenate, 0.5 mg p-aminobenzoic acid, 0.25 mg biotin, 50 mg cycloheximide, 18 g agar (vitamins and cycloheximide were filter-sterilized), 1 L of distilled water adjusted to pH

7.2). The plates were incubated at 28°C for 30 days. The colonies of actinomycetes were taken and transferred on the yeast malt extract agar (YMA, 10 g malt extract, 4 g yeast extract, 4 g glucose, 18 g agar and 1 L distilled water). Isolated actinomycetes were stored in 15% glycerol at −70°C until used.

Antifungal activities of actinomycetes were evaluated on V8 agar medium (20% V8 juice, 18 g agar, 1 L water and pH 6.4 before autoclave) against various plant pathogenic fungi such as *Alternaria mali, Magnaporthe grisea, Fusarium oxysporum* f.sp. *lycopersici, Colletotrichum orbiculare, Rhizoctonia solani* and *Phytophthora capsici.* The actinomycete isolates cultured on YMA slants were streaked in the center of V8 agar plates (9 cm in diameter) and incubated at 28°C for 3 days. Mycelial disks (8 mm in diameter) of various plant pathogenic fungi and oomycete were cut from the growing margin of cultures of pathogens and were placed on the plates 3 cm away from the streak-cultures of the actinomycete isolates. After the plates were incubated at 28°C for 7-10 days, the inhibition zones were measured. The strain JK-1, which showed strong inhibitory activity against plant pathogenic fungi and oomycete was selected for further study.

Analysis of 16S rDNA gene sequence of strain JK-1. Strain JK-1 was grown on YMA medium at 28°C for 7 days. A single colony was cultured in 5 ml LB broth (10 g NaCl, 10 g tryptone, 5 g yeast extract, 1 L water) for 3 days at 28°C in a rotary shaker (200 rpm).

Genomic DNA was isolated from the cultured cells according to the method of Pospiech and Neumann (1995). Strain JK-1 cells were harvested by centrifugation (3000 *g* for 10 min) at room temperature and suspended in 300 μl SET buffer (20 mM Tris-Cl, pH 7.5, 75 mM NaCl, 25 mM EDTA). Lysozyme (1 mg ml^{-1}) was added into the suspension and then incubated at 37°C for 1 h. 1/10 volume of 10% SDS and 0.5 mg/ml proteinase K were added into the solution and incubated with occasional inversion at 55°C for 2 h. Subsequently, it was incubated in 75°C heating block for 20 min. One ml RNase stock (1 μg m^{-1}) was added and incubated at 37°C for 30 min. 1/3 volume of 5 M NaCl and 1 volume of chloroform were added and the preparation was incubated at room temperature for 30 min with frequent inversion. The aqueous layer was separated by centrifugation at 4,500 *g* for 15 min. The chromosomal DNA was precipitated with 1 volume of isopropanol, washed in 70% ethanol (v/v), dried and dissolved in 1 ml of distilled water.

The 16S rDNA sequence of strain JK-1 was amplified by PCR using two universal 16S rDNA primers, FD1 (5'-AGAGTTTGATCCTGGG-3') and RP2 (5'-ACGG-CTACCTT-GTTACGACTT-3') (Weisburg et al., 1991). The PCR reaction mixtures consisted of 100 ng template DNA, 32 μM primer, 0.2 mM dNTPs (Takara, Japan), 1.5 units of *Taq* DNA polymerase (Takara) and 10x *Taq* polymerase buffer (Takara). The PCR amplifications using primers were performed on a PTC-200 (MJ Research, USA). The PCR reaction mixtures were subjected to the initial denaturing step consisting of 95°C for 4 min. The thermal profile used was 35 cycles consisting of 1 min of denaturation at 95°C, 1 min annealing at 58°C and 2 min extension at 72°C. Finally, 8 min extention at 72°C and cooling to 4°C completed the reaction sequence.

Amplified 16S rDNA of strain JK-1 was purified from 1% agarose gel (Wu et al., 1997). The purified PCR products were ligated into the pCR®2.1-TOPO® T vector (Invitrogen™ Co., Carlsbad, CA, USA). Ligated plasmid was transformed into *E. coli* TOP10 cells (Invitrogen™ Co.) by electroporation (E=12.5 kV/cm in 0.2 cm cuvettes). The transformed competent cells were placed on LB-kanamycin agar treated with IPTG and X-gal. Transformants were selected on the basis of the results of the blue-white screening procedure (Sambrook et al., 2001).

Sequencing of 16S rDNA and phylogenetic analysis of 16S rDAN of strain JK-1. 16S rDNA region of strain JK-1 were isolated using Wizard® *plus* SV Minipreps DNA Purification system (Promega, USA). Sequencing of the purified 16S rDNA was performed on a ABI310 automatic DNA sequencer (Applied Biosystems, USA) using Big Dye terminator cycle sequencing ready reaction kits (PE Applied Biosystems, USA). The 16S rDNA sequence analysis was carried out using BLAST network services at the NCBI (Altschul et al., 1997). Sequences were edited in the DNASTAR (Lasergene system for sequence analysis) computer package. An alignment was performed using the CLUSTAL W program (Thompson et al., 1994). The PAUP version 4b10 (Swofford, 2002) software was used for phylogenetic analyses. The resultant data was examined using the maximum-parsimony method (Fitch, 1971) and neighbor-joining method (Saitou and Nei, 1987). The topology of the resulting unrooted tree was evaluated by bootstrap assay of the neighbor-joining tree performed with 1000 replications. Phylogenetic tree display, edit and print were carried out using TreeView program version 1.6.6. (Page, 1996). The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain JK-1 is AY822607.

Analysis of chemotaxonomic characteristic of strain JK-1. In a cell wall preparation, the identification of the diaminopimelic acid type was performed, as described by Becker et al. (1965) and Schaad (1985). Strain JK-1 for the chemical analyses was prepared by culturing in yeast

extract-malt extract broth (ISP 2) for 4 days at 28°C in a rotary shaker (120 rpm). Incubated cells were harvested by centrifugation and filtered through filter paper (Whatman No. 1). Harvested cells were washed with methanol and H_2O. Freeze-dried cells (20 mg) were hydrolyzed with 5 ml of 6 N HCl at 100°C for 18 h. To remove the HCl, the filtrate was evaporated. The isomer type of the diaminopimelic acid of the peptidoglycan layer was analyzed by TLC (Lee and Hwang, 2002) using the modified solvent system of methanol-H_2O-10 N HCl-pyridine (80:26:2.5:20, v/v/v/v) (Becker et al., 1965) and stained with ninhydrine solution (0.1% ninhydrine in acetone, w/v), followed by air-drying and heating at 100°C for 2 min.

Morphological and biochemical characterization of strain JK-1. Spore chain morphology, spore size and surface ornament of strain JK-1 were determined by scanning electron microscopy (SEM). Specimens for SEM were prepared by using the method of Williams and Davies (1967). The strain JK-1 was grown on yeast malt extract agar medium at 28°C for 14 days. The spore morphology was classified as the morphological categories suggested by Pridham et al. (1958).

Cultural characteristics of strain JK-1 was determined by the method of the ISP (International Streptomyces Project) (Shiring and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology (Locci, 1989). Biochemical and physiological characteristics of strain JK-1 were determined as described by Williams et al. (1983a, b).

Optimization of culture conditions for antibiotic production. Four media containing different carbon and nitrogen sources were tested to select the medium and culture time favorable for the antibiotic production. The used media were; glycerol dextrin broth (GDB) (20 g glycerol, 20 g dextrin, 10 g soytone, 3 g yeast extract, 2 g $(NH_4)_2SO_4$, 4 g K_2HPO_4, pH 7.0, 1 L distilled water), glycerol peptone broth (GPB)(20 g glycerol, 10 g polypeptone, 5 g meat extract, pH 7.0, 1 L distilled water), starch casein broth (SCB) (20 g soluble starch, 0.6 g tryptone peptone, 4 g K_2HPO_4, 4 g KNO_3, 4 g NaCl, 1 g $MgSO_4 \cdot 7H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$, 40 mg $CaCO_3$, pH 7.0, 1 L distilled water) and starch glucose broth (SGB) (20 g soluble starch, 10 g glucose, 5 yeast extract, 5 g casamino acid, pH 7.0, 1 L distilled water). Pre-cultured broth (2 ml) of the strain JK-1 was transferred into the 200 ml of four media in 1 L Erlenmeyer flask and then incubated at 28°C for 2-14 days. At different time intervals, the culture filtrates withdrawn from each flask and the cell mass of the strain JK-1 was determined as packed cell volume, which was measured after centrifugation at 3,000 *g* for 10 min. The culture filtrates was loaded on the column packed with Diaion HP-20 resin. The column was washed with 100 ml H_2O three times and then eluted with 100 ml methanol. The methanol eluent were concentrated to 1 ml by rotary evaporator at 38°C and bioassayed against *P. capsici* and *R. solani* using a paper disk assay method.

Production and purification of antifungal substances. Starch glucose broth (SGB) was selected for a large-scale production of antifungal substances. Strain JK-1 was precultured in yeast extract-malt extract broth (200 ml) in a 1 L Erlenmyer flask on a rotary shaker at 150 rpm for 7 days at 28°C. The 3 ml of the pre-cultures was transferred into 300 ml of SGB in a 1 L Erlenmyer flask and incubated at on a rotary shaker at 150 rpm at 28°C for 10 days. The 120 L culture broth was centrifuged at 5000 *g* for 10 min. The culture filtrates were loaded on the column packed with Diaion HP-20 resin. The Diaion HP-20 column was eluted with stepwise gradients of methanol in H_2O (0%, 20%, 40%, 60%, 80% and 100%, v/v). The antifungal activities of all fractions were examined against *C. orbiculare* and *P. capsici* using a paper disk assay method. The antifungal fractions were pooled, concentrated, dissolved in water and extracted with ethyl acetate (5 L). Subsequently, the organic layer was evaporated and dissolved in methanol. The crude extract from the Diaion HP-20 resin column was loaded in an open column packed with C18 resin (Dichroprep RP-18, 40-63 μm, Merck, Germany). The column was eluted with stepwise gradients of methanol and H_2O (0:100, 20:80, 40:60, 60:40, 80:20, 100:0, v/v). Each fraction (3 L) was concentrated *in vacuo* to a small volume and antifungal activity was evaluated against *C. orbiculare* and *P. capsici* using a paper disk assay method. The 80% and 100% methanol fractions were pooled and further purified by silica gel (60F254, 63-200 μm, Merck) open flash column chromatography. The column was eluted with stepwise gradients of chloroform and methanol (100:0, 90:10, 80:20, 70:30, 50:50, 30:70 and 10:90, v/v). Each fraction (3 L) was concentrated, dissolved in methanol and antifungal activity was measured against *C. orbiculare* and *P. capsici* using a paper disk assay method. The 90% and 100% chloroform eluents were selected and chromatographed on a Sephadex LH-20 (Pharmacia, Sweden) column (26 × 950 mm, C26/100, Pharmacia). The column was eluted with methanol at 0.15 ml min^{-1} flow rate. Each fraction (2 ml) was collected by a fraction collector (Pharmacia RediFrac, Pharmacia). All fractions were examined against *C. orbiculare* and *P. capsici.* The antifungal active fractions were combined and subjected to HPLC on a C18 reverse phase column (Symmerty Prep™ C18, 10 μm, 10 × 250 mm, Merck, USA).

Structure elucidation of the antibiotic MA-1. The UV

absorption spectrum of the antibiotic MA-1 was determined with a Beckman DU® 650 spectrometer (Beckman instruments Inc., USA). The structure of the antibiotic MA-1 was elucidated by ^{1}H-NMR, ^{13}C-NMR (Nuclear magnetic resonance) and EI mass spectral analysis. ^{1}H- and ^{13}C-NMR were evaluated in deuterated methanol (CD_3OD).

Evaluations of *in vitro* and *in vivo* antimicrobial activity of the substance MA-1.

***In vitro* antimicrobial activity.** *In vitro* antimicrobial activity of the antifungal substance MA-1 was measured against plant pathogenic fungi, oomycete, yeasts and bacteria. To determine minimum inhibitory concentrations (MIC), oomycete and various fungi were inoculated on a potato dextrose agar media and incubated at 28°C for 7-10 days. These plates were incubated until an extended fungal mat formed spores. Bacteria and yeast were incubated in a nutrient broth (NB). Sterile potato dextrose broth (1 ml) or nutrient broth was transferred into each well of 48-well microtiter dish (Cell Wells ™, Corning Glass Works, Corning, NY, USA). The purified antibiotics in the range of 0 to 500 μgm^{-1} were added into the microwells. The spore suspensions (10^4 spores ml^{-1}) of *A. mali, Botrytis cinerea, Cladosporium cucumerinum, C. orbiculare, F. oxysporum f.sp. lycopersici, M. grisea, Cylindrocarpon destructans, Didymella bryoniae* and *P. capsici* and mycelial suspension of *R. solani* and cell suspensions (10^3 cfu ml^{-1}) of *Sclerotinia sclerotiorum, Bacillus subtilis, Saccharomyces cerevisiae, Ralstonia solanacearum* and *Xanthomonas campestris* pv. *vesicatoria* were prepared as inocula. The 100 μl of inoculum suspension was added to each well containing 100 μl of the purified antibiotics and 200 μl of PDB (or NB). These plates was incubated at 28°C for 2-5 days. The commercial compound phenylacetic acid was purchased from Aldrich Chemical Co. and was examined under identical conditions described above.

Inhibition of conidial spore germination by isolated antibiotics was evaluated in a 24-well microtiter dish (Cell Wells™, Corning Glass Works, USA). The purified antibiotics in the range of 0 to 500 μgm^{-1} were added into the microwells and same volume of conidial suspension (3×10^5 spores ml^{-1}) of *C. orbiculare* and *M. grisea* was transferred to each microwell. The treated microtiter dishes were incubated at 28°C for 5-12 h. The spores germinated were counted on a haemacytometer under a light microscope. These dishes were incubated at 28°C, until the hyphae of the germlings had an extension at the control. Length of hyphal growth was determined by comparison of the hyphal length of the germlings treated with the antibiotic MA-1 and commercial fungicide (tricyclazole, iprobenfos and chlorothalonil) with that of control germlings. The commercial compounds tricyclazole [75% active ingredient (a.i.), Dongbu Hannong Co., Seoul, Korea], iprobenfos (48% a.i., Kyung Nong Co., Seoul, Korea) and chlorothalonil (75% a.i., Kyung Nong Co.) were examined under same conditions described above.

The antifungal substances were identified using the bioautography on TLC plate (Lee et al., 2004). A series of amounts of purified antibiotics and chlorothalonil, both of which were dissolved in methanol, were applied as a spot on a silica gel TLC plates (60F 254, 0.2 mm in thickness, Merck, Germany). After air-drying to remove solvent, the TLC plate was placed on the water agar plate (2% agar, 15 cm-diameter Petri dish). Molten PDA seeded with the 10^8 spores ml^{-1} of *C. orbiculare* and *M. grisea* were spread onto the TLC plate and incubated at 28°C for 3 days. The assay plates were stained with 0.1% naphthol blue black solution in 5% acetic acid for 3 min, and then destained with 5% acetic acid for 2-3 h. Inhibition of mycelial growth was clearly visualized on the blue-black-colored background.

***In vivo* antifungal activity**. The protective activity of the antibiotic MA-1 on cucumber plants against *C. orbiculare* was determined in a greenhouse. Cucumber (*Cucumis sativus* L. cv. Baekrokdadaki) seeds were sown in a plastic pot (10 cm in diameter) containing a steam-sterilized soil mix (peat moss, perlite and vermiculite, 5:3:2, v/v/v) and grown at 28 ± 2°C. The antibiotic MA-1 and the commercial fungicide chlorothalonil dissolved in water were diluted with 0.1% Tween 20 solution to give the concentrations of 10,100, 250, 500 $\mu g\ ml^{-1}$. Each of compound solutions was uniformly spread at the third leaf stage 4 h before inoculation with spore suspension of *C. orbiculare* (10^8 spores ml^{-1}). The inoculated plants were placed in a dew chamber at 28-30°C for 24 h and then transferred to the greenhouse for further incubation. Lesions on the primary or secondary leaves of plants were counted 6 days after inoculation.

The antifungal activity of the antibiotic MA-1 was evaluated against leaf blast of rice. Rice (*Oryza sativa* L., cultivar Ilpoom) was grown in a greenhouse at 28 ± 2°C. The plastic pot (5 × 15 × 10 cm) was filled with the above described soil mix. The commercial fungicides tricyclazole and iprobenfos were used to compare the antifungal activity with the antibiotic MA-1. The antibiotic MA-1 and tricyclazole and iprobenfos dissolved in water were diluted with 0.1% Tween 20 solution to give the concentrations of 10,100, 250 and 500 $\mu g\ ml^{-1}$. Each of compound solutions was uniformly spread on rice plants at the five leaf stage 4 h before inoculation of *M. grisea*. Conidial suspension (10^8 conidia ml^{-1}) was sprayed on the leaves of rice. The inoculated plants were placed in a dew chamber at 28-30°C for 24 h and then transferred to the greenhouse for further

incubation. Lesions on secondary or third leaves of plants were counted 3 days after inoculation.

Statistical analysis was determined using the Statistical Analysis Systems (SAS Institute, USA). The means of all data were analyzed by the least significant difference test (LSD).

Results

Isolation of antibiotic-producing actinomycete strain JK-1 from soil. Various actinomycetes and bacterial colonies appeared on the humic acid vitamin agar after incubation for 30 days. Inhibitory effects of actinomycete colonies were evaluated by measuring inhibition zones against various plant pathogenic fungi and oomycete including *A. mali, M. grisea, F. oxysporum* f.sp. *lycopersici, C. orbiculare, R. solani* and *P. capsici* (data not shown). Among actinomycetes tested, strain JK-1 showed a strong antifungal activity against 6 plant pathogenic fungi and oomycete. Therefore, actinomycete strain JK-1 was selected for a large-scale production and purification of antifungal compounds.

Identification of actinomycete strain JK-1. Spore chain and surface morphology of strain JK-1 were determined by scanning electron microscopy (SEM) (Fig. 1). The non-motile actinomycete strain JK-1 produced spores singly borne on sporophores. Spore surface was smooth. Spores were spherical and 0.9-1.2 μm in diameter.

Cultural characteristics of strain JK-1 was determined on various ISP(International Streptomyces Project) media. The strain JK-1 grew well on yeast extract-malt extract agar (ISP2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), tyrosine agar (ISP 7) and Bennett's agar (data not shown). The aerial mycelium of strain JK-1 was scarcely produced on these media. The aerial mycelium color was dark brown on ISP 2 and 4 media. The substrate mycelium color was dark brown on ISP3 and Bennett's agar. The strain JK-1 produced soluble pigments on ISP 7, but did not on other ISP media. These data of the strain JK-1 were mostly identical to those of *Micromonospora aurnatiaca* DSM 44438.

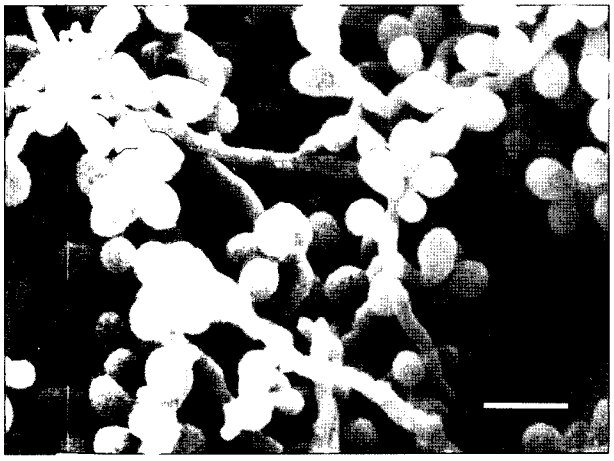

Fig. 1. Scanning electron micrograph of the spores of the actinomycete strain JK-1 cultured on the yeast malt extract agar for 7 days. Bar = 1 μm.

The whole cell wall hydrolysate of strain JK-1 was developed in the cellulose-coated TLC plate. Compared with the standard DAPs (diaminopimelic acids), cell wall extract of the strain JK-1 contained *meso*-diaminopimelic acid (data not shown).

The physiological and biochemical characteristics of strain JK-1 are shown in Table 1. The strain JK-1 did not produce melanin pigment on ISP 6 medium, but it was produced on ISP 7 medium. The strain JK-1 did not degrade tyrosine. Strain JK-1 tolerated well up to 3% NaCl in the basal medium. The strain JK-1 did not grow below 10°C. The strain JK-1 utilized L-cysteine and L-histidine as nitrogen sources, but not DL-α-amino-n-butyric acid, L-hydroxyprolone, L-phenylalanine and L-valine. Strain JK-1 utilized adonitol, arabinose, dextran, fructose, meso-inositol, mannitol, D-melezitose, D-melibiose, raffinose, L-rhamnose, xylitol and xylose as carbon sources. To determine pH range for growth, strain JK-1 was incubated in nutrient broth (NB). It grew well in pH 8. The strain JK-1 showed antibiosis against *Bacillus subtilis* NCIMB 3610, but did not *Aspergillus niger, Candida albicans* CBS562, *Micrococcus luteus* NCIMB 196, *Micrococcus luteus* NCIMB 196, *Saccharomyces cerevisiae* CBS 1171 and *Streptomyces murinus* ISP 5091. Physiological and biochemical properties of the strain JK-1 were 100% identical to those of *M. aurantiaca* DSM 44438. However, the results of *M. fluvopurpurea* DSM 43918 were significantly different from those of strain JK-1 and *M. aurantiaca.*

The almost complete 16S rDNA region (1539 nucleotides) of the strain JK-1 was sequenced (data not shown) and was compared with those of *Micromonospora* species deposited in the GenBank database (NCBI, Bethesda, MD, USA), which indicated that the organism is phylogenetically related to members of the genus *Micromonospora.* In the rooted phylogenetic tree based on the neighbour-joining method, strain JK-1 was placed in the clade with *M. aurantiaca*^{T}, *M. fulvopurpurea* and *M. chersina*^{T} (Fig. 2). Strain JK-1 exhibited comparable levels of similarity of 99.9% (1472/1473), 99.7% (1469/1473) and 97.8% (1440/1473) to the strains of *M. aurantiaca*^{T}, *M. fulvopurpurea* and *M. chersina*^{T}, respectively. These results indicate that strain JK-1 belongs to the *M. aurantiaca.*

Table 1. Physiological and biochemical characteristics of actinomycete strain JK-1, *Micromonospora. aurantiaca* (DSM 44438) and *M. fulvopurpurea* (DSM 43918)

Characteristic	Strain JK-1	*M. aurantiaca* DSM 44438	*M. fulvopurpurea* DSM 43918
Formation of aerial mycelium	−	−	ND[a]
Spore chain morphology *Rectiflexibiles*	−	−	ND
Spore chain morphology *Spirals*	−	−	ND
Melanin production on ISP 7 medium	+	+	ND
Degradation of			
Casein	+	+	+
Esculin	+	+	+
Gelatin	+	+	+
Starch	+	+	+
Tyrosine	−	−	+
Xanthine	+	+	+
Maximum NaCl tolerance (%, w/v)	3%	3%	3%
Oxidase	−	−	−
Catalase	+	+	+
Citrate utilization	−	−	+
Resistance to antibiotics			
Neomycin	−	−	−
Oleomycin	−	−	−
Penicillin G	+	+	+
Rifampicin	−	−	−
Growth temperature range (°C)			
4	−	−	−
30	+	+	+
45	+	+	+
Growth on sole nitrogen source (0.1%, w/v)			
DL-α-amino-n-butyric acid	−	−	+
L-Cysteine	+	+	+
L-Histidine	+	+	+
L-Hydroxyproline	−	−	+
L-Phenylalanine	−	−	+
L-Valine	−	−	+
Growth on sole carbon source (1%, w/v)			
Adonitol	+	+	+
Arabinose	+	+	+
Dextran	+	+	+
Fructose	+	+	+
meso-Inositol	+	+	+
Mannitol	+	+	+
D-Melezitose	+	+	+
D-Melibiose	+	+	+
Raffinose	+	+	+
L-Rhamnose	+	+	+
Xylitol	+	+	+
Xylose	+	+	+
Growth of pH			
5	−	−	−
8	+	+	+
11	+	+	+
13	−	−	−
Antibiosis against			
Aspergillus niger	−	−	−
Bacillus subtilis NCIMB 3610	+	+	−
Micrococcus luteus NCIMB 196	−	−	−
Saccharomyces cerevisiae CBS 1171	−	−	−
Streptomyces murinus ISP 5091	−	−	−

[a]ND. no data.

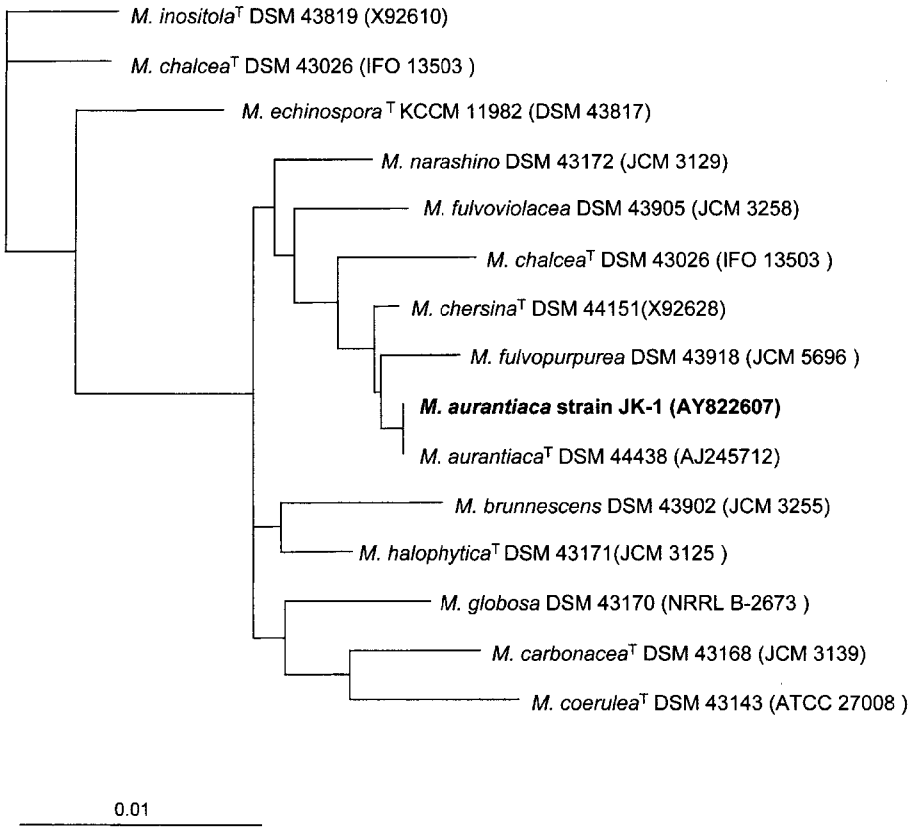

Fig. 2. Phylogenetic tree of actinomycete strain JK-1 based on the partial 16S rDNA sequences of 14 *Micromonospora* strains. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

Media and culture time for antibiotic production. Four media containing different carbon and nitrogen sources were tested to select the medium and culture time favorable for the antibiotic production from the strain JK-1 culture (data not shown). Each of culture filtrates was loading on the open column packed with Diaion HP-20 resin. The methanol eluent was concentrated and evaluated using a paper disk assay method. The culture filtrates of strain JK-1 exhibited inhibitory effect against *P. capsici* and *Rhizoctonia solani*. The culture filtrates from starch glucose broth (SGB) was most effective among those of the four media. The time courses of dry cell weight and pH in the culture of strain JK-1 were examined in four different media (data not shown). The pH in different media was not changed drastically during the culturing of the strain JK-1. After 6 days of incubation, the pH gradually increased in the cultures of GPB and SGB, but decreased in the GDB and SCB. Dried cell weight of the strain JK-1 increased gradually to 10 days after inoculation and then declined drastically, except for the GDB cultures. The SGB cultures showed the strongest antifungal activity among the four different culture filtrates (data not shown). The pH of culture of strain JK-1 gradually increased to 8.5 during the culturing for 14 days. The SGB cultured filtrates were most effective in inhibiting mycelial growth of *P. capsici* and *R. solani*. Therefore, the SGB medium was selected for a large-scale production.

Production and purification of antifungal substances. Total broth cultures (120 L) of strain JK-1 were used for further isolation of antibiotic MA-1. Initial purification of antibiotics was carried out on a Diaion HP-20 column. The antifungal activities of methanol eluates were determined against *P. capsici* and *C. orbiculare* using a paper disk bioassay method. Most of the antifungal activities were recovered from the eluates of 80% and 100% methanol. The 80% and 100% fractions were combined and concentrated. Subsequently, the active fractions were extracted with ethyl acetate and purified by C_{18} reversed-phase flash chromatography. The 80% and 100% methanol fractions were remarkably inhibitory against *P. capsici* and *C. orbiculare*. These extracts were subsequently purified by silica gel open flash column chromatography. The high antifungal activity was detected in the fractions with 90% and 100% chloroform eluates, followed by Sephadex LH-20 column chromatography. The fraction no. 86-127 show-

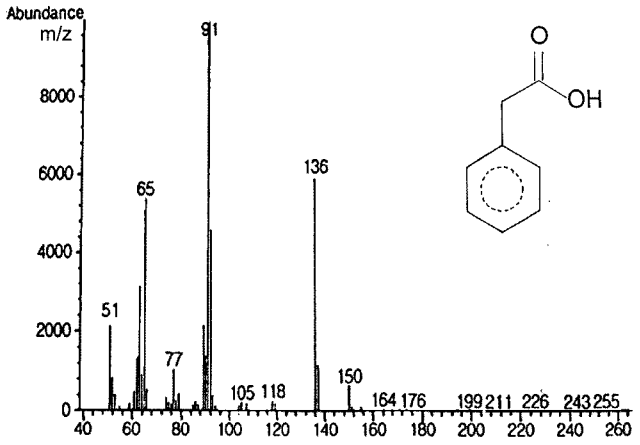

Fig. 3. Low resolution EI mass spectrum of the antibiotic MA-1.

ed antifungal activities against *P. capsici* and *C. orbiculare*. The active fractions were combined and re-chromatographed on Sephadex LH-20 column. Fraction no. 91-118 showed strong antifungal activity and the active fractions were combined. The antibiotic MA-1 showed UV absorption maximum at 223 nm and 258 nm in methanol. Antifungal fractions were subjected to reversed-phase HPLC. The chromatography was performed with a Gilson HPLC system using a linear gradient elution, starting from 30% CH_3CN to 50% CH_3CN in water at a flow rate of 1 ml^{-1}. A pure antibiotic MA-1 was detected at the retention time of 4.98 min.

Structure elucidation of the antifungal substance MA-1 purified from actinomycete strain JK-1. The structure of the antifungal substance MA-1 was elucidated by ^1H-, ^{13}C-NMR and EI mass spectral analysis (Fig. 4). The molecular formula of the antibiotic MA-1 was deduced to be $C_8H_8O_2$ by EI mass analysis, which gave molecular ion at m/z 136 (M+): EI MS m/z 65 (16%), 91 (100%), 92 (20%), and 136 (27%) (Fig. 3). Based on ^1H-NMR analysis in CD_3OD, eight hydrogens were counted including one exchangeable proton. ^1H NMR: δ 3.58 (2H, s) and δ 7.32-7.20 (5H, m) ppm. ^{13}C NMR analysis in the same solvent showed carbon signals at δ 175.56 (C-1), δ 136.07 (C-3), δ 130.34 (C-4, C-8), δ 129.45 (C-5, C-7), δ 127.89 (C-6) and δ 41.94 (C-2) ppm. HMBC analysis revealed that the C-3 (δ 139.41) of the aromatic ring substructure could be connected to the methylene protons (δ 3.58) of C-2 which was in turn connected to the carbonyl carbon (175.56). Theses results revealed that the MA-1 compound has the structure of phenylacetic acid, which were exactly matched to the data from the reference compound (Hwang et al., 2001).

***In vitro* and *in vivo* antifungal activity of the substance MA-1.** *In vitro* antimicrobial activity of the antifungal

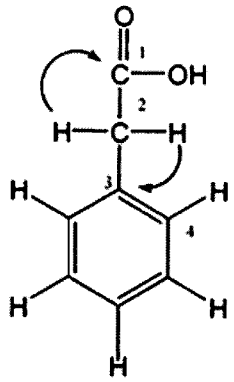

Fig. 4. Structure of the antibiotic MA-1 isolated from *M. aurantiaca* strain JK-1.

Table 2. Minimum inhibitory concentrations (MIC) against various microorganisms of the antibiotic MA-1 and the authentic compound phenylacetic acid (PAA)

Microorganism	MIC (μg/ml)[a]	
	MA-1	PAA
Alternaria mali	50	70
Botrytis cinerea	70	70
Cladosporium cucumerinum	>500	>500
Colletotrichum orbiculare	150	200
Cylindrocarpon destructans	300	>500
Didymella bryoniae	300	300
Fusarium oxysporum f.sp. lycopersici	>500	>500
Magnaporthe grisea	100	100
Phytophthora capsici	70	70
Rhizoctonia solani	150	200
Sclerotinia sclerotiorum	150	300
Bacillus subtilis	350	>500
Candida albicans	150	150
Saccharomyces cerevisiae	70	150
Ralstonia solanacearum	>500	>500
Xanthomonas campestris pv. *vesicatoria*	300	300

[a]The lowest concentration that completely inhibit the growth of microorganisms was examined after incubation for 3-7 days.

substance MA-1 and the authentic compound phenylacetic acid were evaluated against plant pathogenic fungi, oomycete, yeasts and bacteria (Table 2). The antibiotic MA-1 completely inhibited the mycelial growth of *A. mali, Botrytis cinerea, M. grisea P. capsici* and *Saccharomyces cerevisiae* at less than 100 μg ml^{-1}. Growth of *F. oxysporum f.sp. lycopersici, Ralstonia solanacearum* and *Cladosporium cucumerinum* were not inhibited even at over 500 μg ml^{-1}.

The antifungal activity of phenylacetic acid and the commercial fungicides chlorothalonil and tricyclazole were identified using the bioautography on TLC plate (Fig. 5). Mycelial growth of *M. grisea* and *C. orbiculare* were strongly inhibited on the silica gel-coated TLC plates spotted with phenylacetic acid at various concentrations. Phenylacetic acid was strongly as active as tricyclazole and

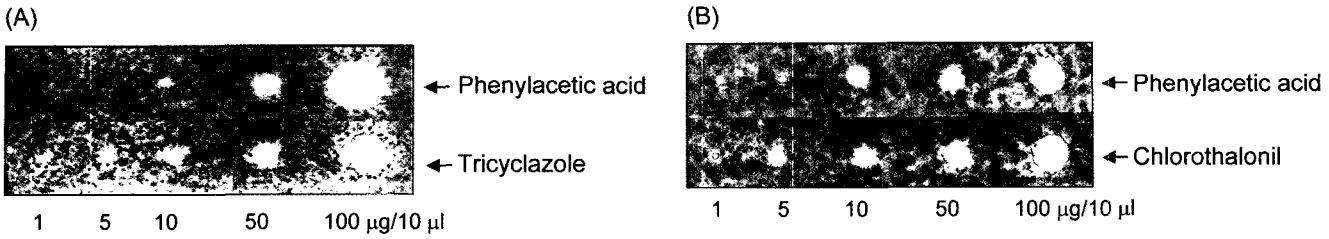

Fig. 5. TLC bioautographs of antifungal activity of phenylacetic acid and commercial compounds against mycelial growth of (A) *M. grisea* and (B) *C. orbiculare* at various concentrations.

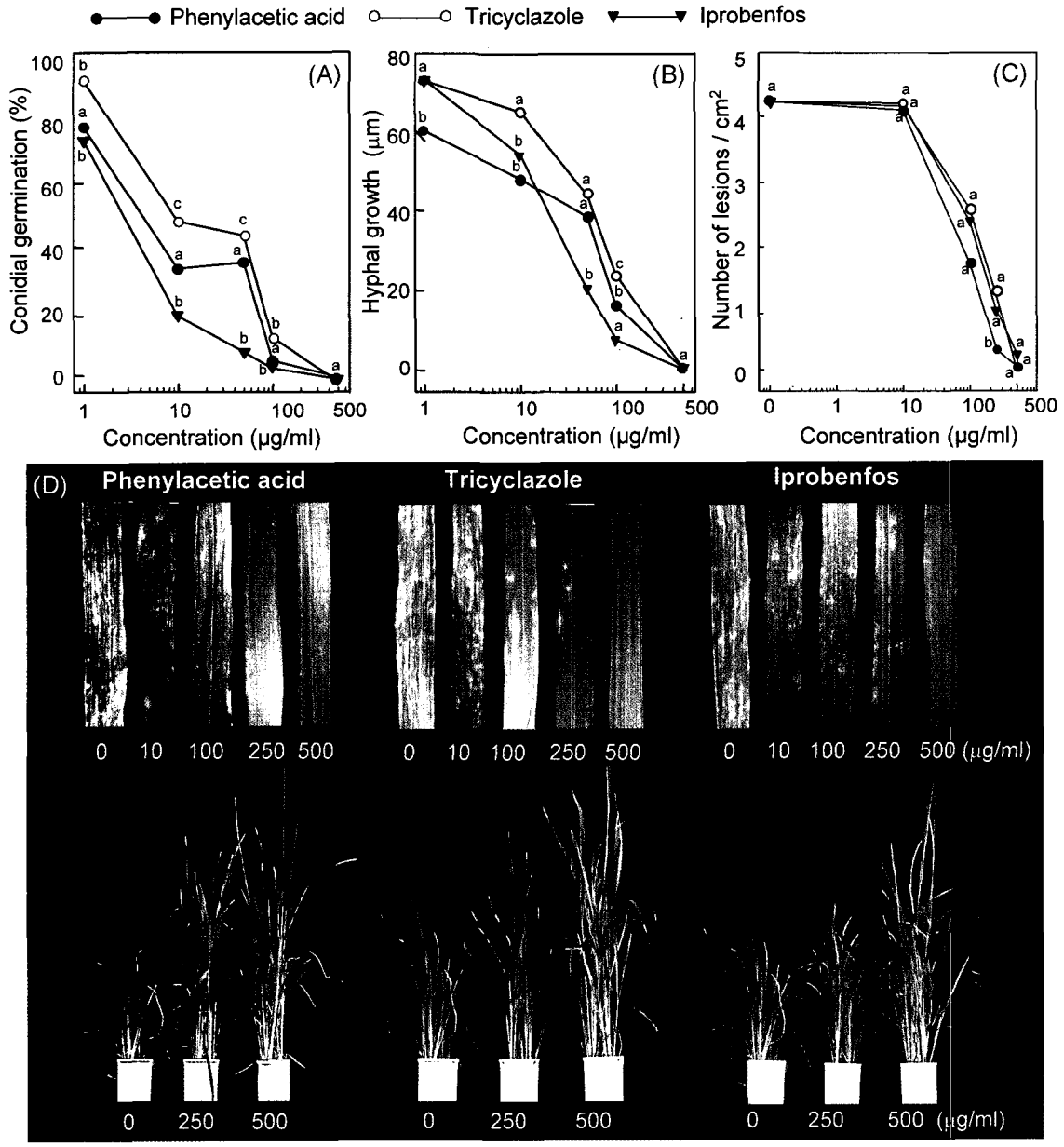

Fig. 6. (A, B) Inhibitory effects of phenylacetic acid, and commercial compounds tricyclazole and iprobenfos on hyphal growth and conidial germination of *M. grisea*. (C) Effects of the phenylacetic acid, tricyclazole and iprobenfos on the development of rice leaf blast at 7 days after inoculation of *M. grisea* (5×10^5 spores ml^{-1}). The lesion numbers were rated after appearance of disease symptoms on control plants. Means at each concentration followed by the same letters are not significantly different ($P = 0.05$) according to the least significant difference test. (D) *In vivo* efficacies of of phenylacetic acid, tricyclazole and iprobenfos for control of rice blast disease were photographed at 7 days after inoculation.

chlorothalonil against mycelial growth of *M. grisea* and *C. orbiculare*, respectively.

Phenylacetic acid and commercial fungicides tricyclazole and iprobenfos completely inhibited conidial germination of *M. grisea* at 100 μg ml^{-1} (Fig. 6A). There was no difference between phenylacetic acid and the commercial compounds in inhibiting conidial germination. Inhibitory effects of phenylacetic acid and commercial fungicides on hyphal growth of *M. grisea* are shown in Figure 6B. Treatments with increasing concentrations of the phenylacetic acid and commercial fungicides significantly inhibited hyphal growth of *M. grisea*, with similar inhibitory effects in the three compounds, and the complete inhibition was ascertained at 500 μg ml^{-1} of all three compounds.

The phenylacetic acid and commercial fungicide tricyclazole and iprobenfos were evaluated under a greenhouse condition for its ability to control leaf blast of rice caused by *M. grisea* (Fig. 6C and 6D). As the concentration of the phenylacetic acid and the commercial fungicides increased, *M. grisea* infection was inhibited on rice leaves. Treatment with 250 μg ml^{-1} of phenylacetic acid and tricyclazole and iprobenfos greatly recuced blast lesions on rice leaves. Complete control efficacy was accomplished by treatment with 500 μg ml^{-1} of all three compounds. *In vivo* efficacy of phenylacetic acid for control of rice blast was similar to that of tricyclazole and iprobenfos.

Phenylacetic acid and the commercial fungicide chlorothalonil inhibited conidial germination of *C. orbiculare* at 100 μg ml^{-1} and 500 μg ml^{-1}, respectively (Fig. 7A). In addition, bothe compounds also inhibited hyphal growth of

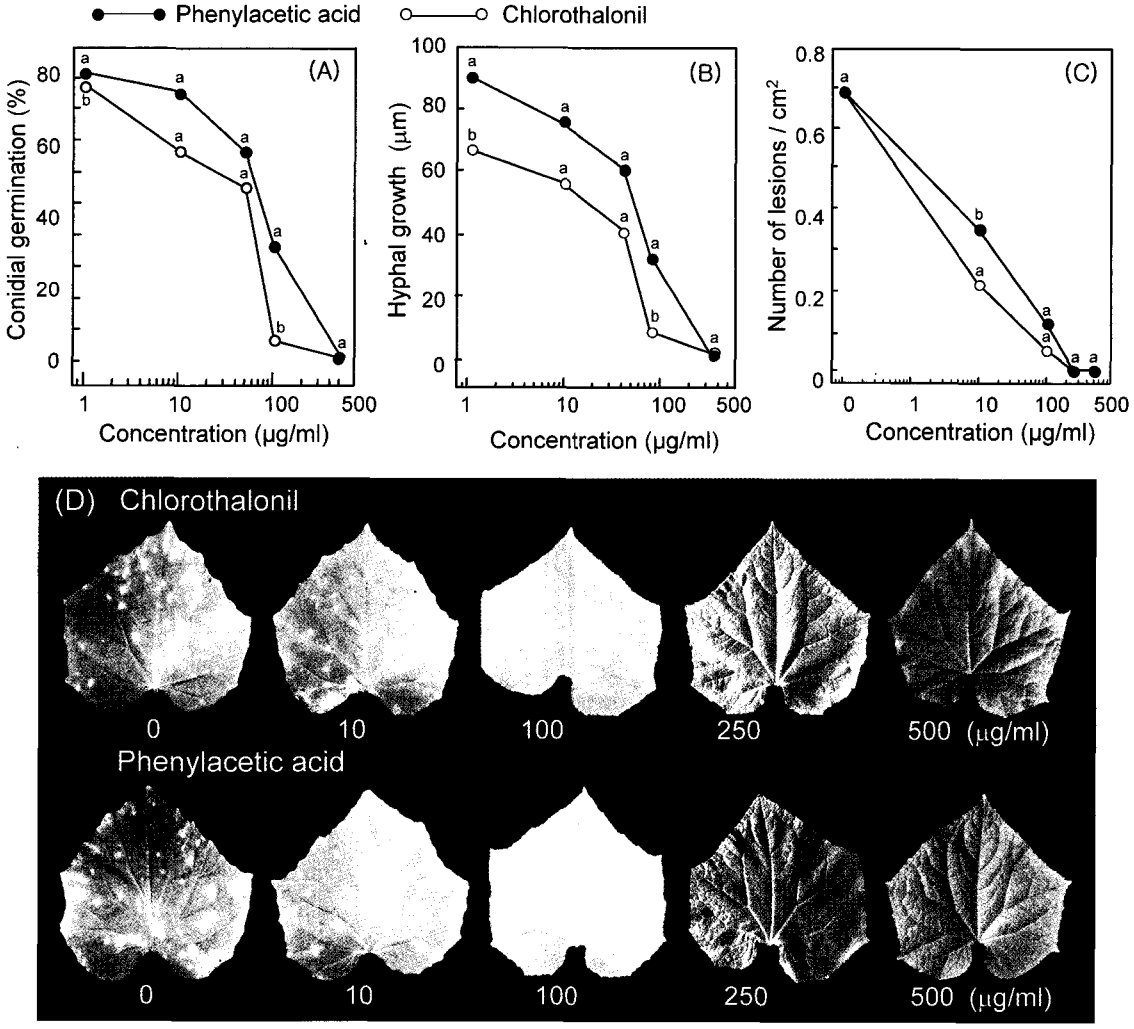

Fig. 7. (A, B) Inhibitory effects of phenylacetic acid and commercial compound chlorothalonil on conidial germination and hyphal growth of *C. orbiculare*. (C) Effects of the phenylacetic acid and chlorothalonil on anthracnose development at 10 days after inoculation of *C. orbiculare* (10^6 spores ml^{-1}). The lesion numbers were rated after appearance of disease symptoms on control plants. Means at each concentraion followed by the same letters are not significantly different ($P = 0.05$) according to the least significant difference test. (D) *In vivo* efficacies of phenylacetic acid and chlorothalonil for control of anthracnose disease were photographed at 10 days after inoculation.

C. orbiculare (Fig. 7B). Phenylacetic acid completely inhibited hyphal growth at 500 μg ml^{-1}, whereas complete inhibition of the hyphal growth was achieved by treatment with 100 μg ml^{-1} of chlorothalonil. *In vivo* efficacies of the phenylacetic acid and chlorothalonil for control of anthracnose disease on cucumber plants are presented in Figures 7C and 7D. Treatment with 100 μg ml^{-1} of phenylacetic acid and chlorothalonil drastically reduced number of lesions on cucumber leaves. No anthracnose symptoms were found on the cucumber leaves treated with 250 μg ml^{-1} (Fig. 7D). Phenylacetic acid and chlorothalonil did not induce any phytotoxicity on cucumber plants.

Discussion

Isolation and identification of *M. aurantiaca* JK-1. The actinomycetes are well known as a novel source of industrially useful metabolites such as antibiotics. The actinomycetes exist in most soils. The actinomycete strain JK-1 was isolated from a soil sample of Joung-bal Mountain in Ko-yang, Korea. The actinomycete strain JK-1 was tested for antifungal activities against various plant pathogenic fungi and oomycetes, such as *P. capsici, M. grisea, C. orbiculare, Fusarium oxysporum* and *A. mali.* The actinomycete strain JK-1 selected for a large-scale production and purification had strong antifungal activity against various plant pathogenic fungi. Actinomycete strain JK-1 was identified based on the morphological, physiological and biochemical characteristics, and 16S rDNA gene analysis. The actinomycete strain JK-1 produced spores singly borne on sporophores branched from substrate hyphae and little aerial mycelium. Spores were spherical and non-motile. The spore surface of the actinomycete strain JK-1 was found to be smooth. The cell wall of the actinomycete strain JK-1 contains *meso*-diaminopimelic acid. *meso*-Diaminopimelic acid represents a major taxonomic constituent of the murein of the genus *Micromonospora* (Kawamoto et al., 1981). Based on the DAP type and morphological characteristics examined by SEM (scanning electron microscopy), the strain JK-1 was confirmed to belong to the genus *Micromonospora.* Cultural, physiological and biochemical characteristics of *Micromonospora* sp. strain JK-1 were compared to the characteristic data of reference strains *M. aurantiaca* (DSM 44438) and *M. fulvopurpurea* (DSM 43918). The characteristics of strain JK-1 were best matched with those of *M. aurantiaca* (DSM 44438). Molecular properties were employed in the classification, identification, and recognition of phylogenetic relationships among actinomycetes (Korn et al., 1978). The 16S rDNA gene analysis revealed that the strain JK-1 belongs to *M. aurantiaca.* Therefore, the strain JK-1 was identified as *M. aurantiaca.*

Isolation and antifungal activity of phenylacetic acid from the cultural filtrates of the strain JK-1. The genus *Micromonospora* spp. known to be widely distributed in soils of various geographical regions (Vobis, 1991) has been recognized as one of the important sources for antimicrobial metabolites. *M. aurantiaca* strain JK-1 showed antifungal activity against *P. capsici, M. grisea, C. orbiculare, Rhizoctonia solani, Fusarium oxysporum* and *A. mali.* Therefore, *M. aurantiaca* strain JK-1 was selected for a large-scale production and purification of antifungal compounds. Antibiotics are usually produced by actinomycetes cultured in nutritionally rich media, although sometimes favorably produced in nutritionally limited media (Okami and Hotta, 1998). Antibiotic production is regulated by many factors such as carbon and nitrogen sources, inorganic phosphate, trace salts and bioregulators. Among them, carbon and nitrogen catabolites have been known to be major factors in biosynthesis of antibiotics (Demain, 1981). In this paper, the antifungal activity of *M. aurantiaca* strain JK-1 was examined in the four media containing different carbon and nitrogen sources. Optimal medium and culture time for producing antibiotics from the strain JK-1 also were examined to determine the best condition for large-scale production of antibiotics. The abundant antibiotic was produced in the culture filtrates from starch glucose broth (SGB) and favorable culture time was 10 days after incubation. Therefore, large scale fermentation was carried out in SGB for 10 days at 28°C.

The antibiotic MA-1 that showed antifungal activity was purified from the culture extracts of *M. aurantiaca* strain JK-1 using various chromatographic procedures. The antibiotic MA-1 was purified using Diaion HP-20 column chromatography, C18 reversed-phase flash column chromatography, silica gel flash column chromatography and Sephadex LH-20 column chromatography. Each chromatography was eluted with a stepwise gradient of each organic solvent. All the fractions were tested for their activity against the plant pathogens. Finally, we performed high performance liquid chromatography and the antibiotic compound MA-1 was obtained from a single peak at a retention time of 4.98 min at 220 nm. The purified antibiotic compound MA-1 (399 mg) was a pale yellow powder soluble in methanol.

The structure of the compound MA-1 was elucidated by ^{1}H-, ^{13}C-NMR and EI mass spectral analysis. The structure of antifungal substance MA-1 was determined to be phenylacetic acid based on all the spectral data, which was exactly matched to the data from the reference compound (Hwang et al., 2001). Phenylacetic acid is aerobically catabolized through a set of enzymatic reactions known as the PAA pathway. The *paa* genes, that code for the PAA pathway have been completely identified and characterized

in *E. coli* W (Ferrández et al., 1998), *Azoarcus evansii* (Mohamed et al., 2002; Rost et al., 2002) and two species of *Pseudomonas*, namely *Pseudomonas putida* U (Olivera et al., 1998) and *Pseudomonas* sp. strain Y2 (Alonso et al., 2003). Antifungal activity of phenylacetic acid has earlier been demonstrated by Burkhead et al. (1998) and Hwang et al. (2001, 2004). In particular, Ismet et al. (2004) have discovered phenylacetic acid as an antifungal metabolite from *Micromonospora* sp. M39. *Micromonospora* sp. M39 was identified as a producer of antifungal substances, which showed antifungal activity against the rice blast pathogen *Pyricularia grisea* MPO 293 (Ismet et al., 2004). However, to our knowledges, it is the first report that the *M. aurantiaca* produces phenylacetic acid.

In our study, *in vitro* antimicrobial activity of phenylacetic acid (the antibiotic MA-1) was evaluated against fungi, oomycete, yeasts and bacteria using MICs (minimum inhibitory concentrations), inhibition of spore germination and hyphal growth of *M. grisea* and *C. orbiculare*. TLC bioautography also was performed on the silica-coated TLC aluminum sheet. The MIC is used as an estimate of the antifungal activity of the antibiotics. The antibiotic MA-1 inhibited the mycelial growth of *A. mali*, *Botrytis cinerea*, *M. grisea*, *P. capsici* and *Saccharomyces cerevisiae* at less than 100 μg ml^{-1}, whereas *F. oxysporum f.sp. lycopersici*, *Ralstonia solanacearum* and *Cladosporium cucumerinum* were not affected even at over 500 μg ml^{-1}. There was no difference in antifungal activity between phenylacetic acid and antibiotic MA-1 from *M. aurantiaca* strain JK-1. Phenylacetic acid and commercial fungicides tricyclazole and iprobenfos also effectively inhibited conidial germination and hyphal growth of *M. gisea* and *C. orbiculare*. Treatment with 100 μg ml^{-1} of the phenylaccetic acid and tricycalzole and iprobenfos was effective in inhibiting conidial germination. The 100 μg ml^{-1} of the iprobenfos greatly inhibited hyphal growth. Phenylacetic acid and tricyclazole completely inhibited hyphal growth of *M. grisea* at 500 μg ml^{-1}. Phenylacetic acid began to show inhibitory activity of conidial germination of *C. orbiculare* at the concentration of 500 μg ml^{-1}; however chlorothalonil strongly inhibited conidial germination of *C. orbiculare* at 100 μg ml^{-1}. The phenylacetic acid effectively inhibited hyphal growth of *C. orbiculare* at the concentration of 500 μg ml^{-1} whereas treatment with 100 μg ml^{-1} of the chlorothalonil substantially inhibited hyphal growth.

The control efficacy of phenylacetic acid against *P. capsici* infection on pepper plants was evaluated (Hwang et al., 2001). In this paper, the phenylacetic acid and the commercial fungicides tricyclazole, iprobenfos and chlorothalonill were applied to examine the *In vivo* efficacy for the control of leaf blast on rice leaves and anthracnose on cucumber leaves, respectively, under the greenhouse condition. Phenylacetic acid and commercial fungicides tricyclazole and iprobenfos began to show protective activity against *M. grisea* infection at the concentration of 250 μg ml^{-1}. They did not induce any phytotoxic symptoms on rice leaves even when treated with 500 μg ml^{-1}. The phenylacetic acid and the commercial compound chlorothalonil were compared for the control of anthracnose in cucumber plants. Treatment with 100 μg ml^{-1} of the chlorothalonil greatly inhibited disease development on cucumber leaves. Moreover, no disease symptoms were found on the cucumber leaves treated with each 250 μg ml^{-1} of phenylacetic acid and chlorothalonil.

We could conclude that the phenylacetic acid produced by *M. aurantiaca* strain JK-1 has a strong potent *in vitro* control efficacy against some plant pathogenic fungi such as *P. capsici, C. orbiculare* and *M. grisea*. *In vivo* control efficacy of phenylacetic acid was pronounced against leaf blast on rice and anthracnose on cucumber. For the practical use of the antibiotic MA-1 (phenylacetic acid) as an agricultural fungicide, the disease control efficacy should be further evaluated in the field.

Acknowledgements

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