A Bacterial Endophyte, *Pseudomonas brassicacearum* YC5480, Isolated from the Root of *Artemisia* sp. Producing Antifungal and Phytotoxic Compounds

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An endophytic bacterial strain YC5480 producing antifungal and phytotoxic compounds simultaneously was isolated from the surface sterilized root of *Artemisia* sp. collected at Jinju area, Korea. The bacterial strain was identified as a species of *Pseudomonas brassicacearum* based on its 16S rRNA gene sequence analysis and physiological and biochemical characteristics. The seed germination and growth of monocot and dicot plants were inhibited by culture filtrate (1/10-strength Tryptic Soy Broth) of the strain. The germination rate of radish seeds in the culture filtrate differed in various culture media. Only 20% of radish seeds germinated in the culture media of 1/2 TSB for 5 days incubation. Mycelial growth of fungal pathogens, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* and *Phytophthora capsici* was also inhibited by the culture filtrate of the strain YC5480. An antifungal compound, KS-1 with slight inhibitory activity of radish seed germination at 1,000 ppm and a seed germination inhibitory compound, KS-2 without suppression of fungal growth were produced simultaneously in TSB. The compounds KS-1 and KS-2 were identified to be 2,4-diacetylphloroglucinol (DAPG) and 2,4,6-trihydroxyacetophenone (THA), respectively.

**Keywords**: antifungal, 2,4-diacetylphloroglucinol, *Pseudomonas brassicacearum*, phytotoxic, 2,4,6-trihydroxyacetophenone

Endophytic bacteria have been isolated from a variety of plants and shown to have a number of beneficial effects on the host plant. The associations between bacteria and hosts such as increase of nitrogen fixation, improvement of seed germination and seedling growth, enhancement of nutrient availability, and biocontrol of fungal infections have been reported extensively (Compant et al., 2005; Elbeltagy et al., 2001; Kuklinsky-Sorbé et al., 2004; Nejad and Johnson, 2000; Rosenblueth and Esperanza, 2006). Many isolates of *Pseudomonas* exist within the plant as endophytes, gain some physical protection and actively interact with the host plant for the benefit of both organisms (Rosenblueth and Esperanza, 2006). The strains of *Pseudomonas fluorescens*, *P. corrugata* and *P. putida* were most frequently isolated as endophytes and rhizosphere bacteria (Garbeva et al., 2001; Slininger and Shea-Andersh, 2005; Suzuki et al., 2003).

In many cases, these bacteria are known to be beneficial to the host organisms and even potential sources of plant disease resistance against bacterial and fungal pathogens (Benhamou et al., 1996; Misaghi and Donndelinger, 1990; Ryu et al., 2003). However, it was frequently found that some endophytic bacteria including *Pseudomonas* spp. also damage to the host plants by inhibiting seed emergence, plant height and the growth of plant seedlings, possibly through the production of certain metabolites (van Peer et al., 1990; Sturz and Christie, 1996).

A biological control agent, *Pseudomonas fluorescens*, against wheat take-all root disease has been observed to produce two metabolites, antifungal compound, 2,4-diaceylphloroglucinol (DAPG) and 2,4,6-trihydroxyacetophenone (THA) that is possibly a precursor of DAPG in cultivation liquid medium (Slininger and Shea-Andersh, 2005). The production of other antifungal compound, phenazine-1-carboxylic acid (PCA) has been implicated in the phytotoxicity of *P. fluorescens* on the germination of treated wheat seeds (Slininger et al., 1996; Thomashow and Weller, 1988). A strain of *Pseudomonas chlororaphis* O6 which inhibits the growth of *Fusarium culmorum* and induces systemic disease resistance against a bacterial soft-rot pathogen, *Erwinia carotovora* subsp. *carotovora* by production of PCA also inhibited seed germination of wheat and barley seeds by seed coating (Kang et al., 2007). The high concentration of the phytotoxic compounds in production cultures of the biological control agents induces phytotoxicity in treated...
plant seeds. Thus it may be very important to minimize the production of phytotoxic metabolites by optimization of carbon and nitrogen sources for the development of manufacturing strategies to supply commercial inoculants (Slininger et al., 1996; Slininger and Shea-Andersh, 2005).

In this study we isolated an endophytic bacterium YC5480 showing both antifungal and phytotoxic activities during screening of biocontrol agents against fungal diseases. We describe here the identification and activity of the strain and characterization of the antifungal and phytotoxic substances produced by this strain.

Materials and Methods

Isolation of endophytic bacteria. In the process of screening biological control agents for plant-fungal pathogens, endophytic bacteria were isolated from the plant samples, especially roots of Artemisia sp. collected at Jinju area, Korea (Table 1). The roots were rinsed with tap water and surface sterilized with 1.0% NaOCl for 10 min and with 70% ethanol for about 10 seconds. Root pieces were rinsed again in sterile distilled water (DW), blotted dry on sterile filter paper, placed on 1/10-strength Tryptic Soy Agar (TSA, Difco) and incubated at 28°C for bacterial growth to check the surface contamination. After confirmation of the surface sterility of root segments, 1.0 g of dried plant root was ground in 9.0 ml of phosphate buffer (pH 7.2) with a sterile mortar and pestle. Serial dilutions were made using phosphate buffer and 100 µl of 10^{-10} dilutions was applied on the 1/10 TSA plates and incubated at 28°C for 3 days (McInroy and Kloeper, 1995). Among growing bacterial colonies, only bacterial strains having antifungal and/or seed germination inhibitory activity were selected and maintained on 1/10 TSA at 4°C for further experiments.

Antifungal activity tests. For the selection of the bacterial strains with antifungal activity, confrontation bioassay was used. The agar plug (5 mm) of actively growing fungal pathogens was placed in the center of the 1/10 TSA and Potato Dextrose Agar (PDA, Difco) media with streaking of bacterial isolates around the fungal plug at the same distance. Antifungal activity of culture filtrate or purified compounds was also tested by confrontation bioassay using paper disks (5 mm) loaded with 200 µl of the culture filtrate or solutions of compounds on PDA media. The culture filtrate of strains was prepared using 1/10-strength Tryptic Soy Broth (TSB, Difco). Each strain isolated from 1/10 TSA plates was inoculated in 3 ml broth of 1/10 TSB and incubated for 3 days at 30°C on a rotary shaker (approximately 150 rpm). The cell suspension was centrifuged at 7,520 × g for 15 min, and then the supernatant was filtered through 0.45 µm Minisart syringe end filters (Sartorius, UK & Ireland) for the activity tests. The antifungal activity against Colletotrichum gloeosporioides KCTC 6169, Fusarium oxysporum KCTC 16909, and Phytophthora capsici KACC 40157 was examined after 5 days of incubation at 30°C by measuring zones of inhibition between the edge of the bacterial colony and growing fungal mycelia on PDA. The strains having KCTC and KACC accession numbers were obtained from the Korean Collection for Type Cultures (KCTC), Daejeon, Korea and Korea Agricultural Culture Collection (KACC), Suwon, Korea.

Seed germination inhibitory activity assay. For the test of seed germination inhibitory activity, the culture filtrate was applied to radish (Raphanus sativus) seeds surface-sterilized with 1% NaOCl for 5 min. Five seeds of radish in 3 replications were placed on a piece of sterile Kim-Wipe tissue (2 × 2 cm) soaked with 0.5 ml culture filtrate in a plastic plate. The plates were incubated for 3-5 days at room temperature under normal day light. The percent ratio of radish seed germination and the growth of sprouts were measured. Following crop seeds were treated with the culture filtrate for 5 days at room temperature as described previously to know the seed germination inhibition spectrum of the bacterial filtrate; wheat (Triticum aestivum), barley (Hordeum vulgare), rice (Oryza sativa), soybean (Glycine max), pepper (Capsicum annuum) and lettuce (Lactuca sativa). All crop seeds were purchased from Nongwoo Bio Co. Ltd., Korea.

Biochemical and physiological characterization of a bacterial strain. Among endophytic bacterial strains, the strain YC5480 (KCTC 10957BP) having antifungal and seed germination inhibitory activities was identified on the basis of biochemical, physiological and molecular characteristics according to “Current Protocols in Molecular Biology” (Ausubel et al., 1995) and “Chemical Methods in Prokaryotic Systems” (Goodfellow and O’Donell, 1994). Carbohydrate assimilation was determined by using API 32E and API ID32 STREP at 30°C according to the instructions of the manufacturer (bioMérieux). Cell morphology was observed under a transmission electron microscope (Hitachi, model H-600) with cells grown for one day at 28°C on 1/10 TSA.

DNA extraction and phylogenetic analysis of 16S rRNA gene sequence. Extraction of genomic DNA from the strain YC5480 was done using commercial genomic DNA extraction kit (Core Biosystem, Korea). The 16S rRNA gene was PCR amplified from a small amount of purified genomic DNA by using a set of primers 27F and 1492R and the obtained PCR product was purified and sequenced according to Chung et al. (Chung et al., 1999). The 16S
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Selection of optimal medium for the production of active metabolites. To select culture media for mass production of antifungal and germination inhibitory compounds, the following culture media were used: 1/2-strength TSB, glucose starch broth (GSB) (soluble starch, 5.0 g; glucose, 5.0 g; aspartic acid, 0.5 g; K$_3$HPO$_4$, 0.5 g; MgSO$_4$, 0.5 g and FeSO$_4$, 0.01 g in one liter DW), soybean meal flour medium (SMF) (yeast extract, 4.0 g; beef extract, 1.0 g; soluble starch, 20.0 g; soybean meal, 25.0 g; glucose, 5.0 g; K$_2$HPO$_4$, 0.1 g; NaCl, 2.0 g in one liter DW), soytone glucose broth (SGB) (glucose, 15.0 g; soytone, 15.0 g, yeast extract, 5.0 g; casamino acid, 1.0 g; K$_2$HPO$_4$, 0.1 g; NaCl, 2.0 g and FeSO$_4$·(NH$_4$)$_2$SO$_4$·6H$_2$O·0.05 g and MgSO$_4$·7H$_2$O·0.2 g in one liter DW), Czapek-dox broth (CDB) (saccharose, 30.0 g; NaNO$_3$, 3.0 g; K$_2$HPO$_4$, 1.0 g; MgSO$_4$, 0.5 g; KCl, 0.5 g and FeSO$_4$, 0.01 g in one liter DW) and M523 broth (sucrose, 1.0 g; casamino acid, 8.0 g; yeast extract, 4.0 g and MgSO$_4$, 0.3 g in one liter DW). Each medium (500 ml) was inoculated with the 5 ml culture inoculum of bacterial cells grown overnight and incubated for 3 days at 30°C on a rotary shaker (approximately 150 rpm).

Isolation of antifungal and seed germination inhibitory compounds. After 5 days shaking incubation of the strain YC5480 in 1/2 TSB, the culture broth (10 liters) was centrifuged at 7,520 × g at 4°C for 15 min to remove the bacterial cells. The cell-free supernatant was concentrated under reduced pressure to give 2.5 g of brown oil. The oil was further separated by silica gel MPLC [LiChroprep Si 60, Merck, hexane-EtOAc-i-PrOH (60:5:1), 5 ml/min, detection at 254 nm] to yield compounds, KS-1 (250 mg) and KS-2 (50 mg).

Characterization of antifungal and phytotoxic compounds. UV spectrum was recorded on a Agilent 8453 spectrophotometer and IR spectrum on a Bruker IFS-66/S FTIR spectrometer. ESIMS was obtained on a Agilent 1100LC/MSD trap SL mass spectrometer and HRESIMS was obtained on a high resolution tandem mass spectrometer (JMS-HX110/110A). NMR spectra were measured on a Varian UNITY 500 spectrometer working at 500 MHz for proton and 125 MHz for carbon. The 1H and 13C NMR chemical shifts were referred to CD$_3$OD observed at 3.30 ppm and 49.0 ppm, respectively.

GenBank accession. The GenBank accession number for 16S rRNA gene sequence of strain YC5480 (= KCTC 10957BP) is EU195810.

Results

Identification of the strain YC5480. The bacterial strain YC5480 showing antifungal activity against C. gloeosporioides and germination inhibitory activity against radish seeds was isolated from the surface sterilized root of Artemisia sp. (Table 1). The strain was identified as a

| Strains   | Locality | Host plant          | Inhibition zone of mycelial growth (mm)$^a$ | Inhibition of radish germination$^b$ |
|-----------|----------|---------------------|---------------------------------------------|-------------------------------------|
| YC5355    | Sacheon  | Green tea           | 2-3                                         | 1-2                                 |
| YC54356   | Sacheon  | Garlic              | 1-2                                         | 3                                   |
| YC5480    | Jinju    | Artemisia           | 5-6                                         | 9-12                                |
| YC5497    | Hadong   | Lettuce             | –                                           | 2                                   |
| YC5501    | Jinju    | Lettuce             | 2-3                                         | –                                   |
| YC5507    | Sanchung | Chinese Cabbage     | –                                           | 1-2                                 |
| YC5554    | Sanchung | Pepper              | 2-3                                         | –                                   |

$^a$Impedivity of mycelial growth was tested by confrontation bioassay at 28°C for 5 days incubation.

$^b$The culture filtrate was used to test the radish seed germination inhibitory activity. Five seeds of radish in three replications were placed on a piece of sterile Kim-Wipe tissue (2 × 2 cm) in a plastic plate loaded with 0.5 ml culture filtrate for 3-5 days under normal day light. −: no inhibition; +: weak inhibition; ++: strong inhibition.

$^a$TSA=Tryptic Soy Agar; PDA=Potato Dextrose Agar.
species of *Pseudomonas brassicacearum* based on biochemical and physiological characteristics and its 16S rRNA gene sequence and phylogenetic analyses (Fig. 1). The 16S rRNA gene sequence of the strain was most closely related to species of the genus *Pseudomonas* (< 94.0 – 99.0% sequence similarity), especially to *P. brassicacearum* CFBP 11706T (99.1%), *P. thivervalensis* CFBP 11261T (99.4%), *P. kilonensis* 520-20T (99.4%) and *P. putida* PC36T (99.1%). Some other physiological and biochemical characteristics of the strain YC5480 were also very similar to *P. brassicacearum* which are given as follows. Gram-negative motile rods, 1.0-2.5 µm in length and about 0.5 µm in diameter. Forms mucoid colonies with regular margins when grown on TSA and produces brown-orange diffusible pigment at late stationary phase. Growth is strictly aerobic and catalase-positive. It can use 5-bromo-4-chloro-3-indolyl-N-acetyl-β-D-glucosaminide, and D-sorbitol as a single carbon source, but cannot use the following chemicals; L-arabitol, galacturonic acid, 5-bromo-3-indoxyl-N-acetyl-β-D-glucosaminide, and D-sorbitol as a single carbon source, but cannot use the following chemicals; L-arabitol, galacturonic acid, 5-bromo-3-indoxyl-N-acetyl-β-D-glucosaminide, and D-sorbitol as a single carbon source, but cannot use the following chemicals; L-arabitol, galacturonic acid, 5-bromo-3-indoxyl-N-acetyl-β-D-glucosaminide, and D-sorbitol as a single carbon source, but cannot use the following chemicals; L-arabitol, galacturonic acid, 5-bromo-3-indoxyl-N-acetyl-β-D-glucosaminide, and D-sorbitol as a single carbon source, but cannot use the following chemicals; L-arabitol, galacturonic acid, 5-bromo-3-indoxyl-N-acetyl-β-D-glucosaminide, and D-sorbitol as a single carbon source, but cannot use the following chemicals; L-arabitol, galacturonic acid, 5-bromo-3-indoxyl-N-acetyl-β-D-glucosaminide, and D-sorbitol as a single carbon source, but cannot use the following chemicals; 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Antifungal and phytotoxic endophyte of culture filtrate in inhibiting seed germination and shoot growth of radish was checked with different dilutions. The culture filtrate without dilution inhibited the shoot growth of radish completely (100%) and still had inhibitory activity with 100 times dilution (Fig. 2).

**Antifungal and seed germination inhibitory activity of the purified substances.** The two substances, KS-1 and KS-2 purified from the culture filtrate of *P. brassicacearum* YC5480 exhibited antifungal and seed germination inhibitory activities. The compound KS-1 inhibited mycelial growth of *C. gloeosporioides*, *F. oxysporum*, and *P. capsici* at 100 and 1,000 ppm and also inhibited seed germination of radish slightly at the same concentration. In contrast to KS-1, the compound KS-2 showed only inhibitory activity of seed germination of radish at 100 and 1,000 ppm (Table 4).

**Identification of the active substances.** Substance KS-1 was isolated as colorless oil which analyzed for C_{10}H_{10}O_{5} by combined FABMS and 13C NMR spectrometry. IR absorptions at 3,413 (broad) and 1,624 cm\(^{-1}\) revealed the presence of hydroxyl(s) and ketonic carbonyl(s), respectively. The

| Culture Broth | Germination (%) |
|---------------|-----------------|
|               | Days 1 | Days 3 | Days 5 |
| 1/2 TSB       | 20   | 20   | 20   |
| MS23          | 100  | 100  | 100  |
| SGB           | 30   | 60   | 70   |
| SMF           | 30   | 40   | 40   |
| GSB           | 100  | 100  | 100  |
| CDB           | 10   | 30   | 30   |
| DW            | 100  | 100  | 100  |

*The culture filtrate was used to test the inhibitory activity of radish seed germination. Five seeds of radish in three replications were placed on a piece of sterile Kim-Wipe tissue (2 × 2 cm) in a plastic plate loaded with 0.5 ml culture filtrate for 3-5 days under normal day light. TSBD, Tryptic soy broth; SMF, Soybean meal flour broth; SGB, Soy-tone glucose broth; GSB, Glucose starch broth; CDB, Czapek-dox broth. Distilled water (DW) was used as control.

**Inhibition of mycelial growth of fungal pathogens and radish seed germination by purified compounds KS-1 and KS-2 from the culture filtrate of *Pseudomonas brassicacearum* YC5480**

| Compound | Conc. (ppm) | Colletotrichum gloeosporioides | Fusarium oxysporum | Phytophthora capsici | Inhibition of radish seed germination (%) |
|----------|-------------|--------------------------------|--------------------|---------------------|------------------------------------------|
| KS-1     | 10          | 0                              | 2                  | 0                   | 7                                        |
|          | 100         | 0                              | 4                  | 7                   | 7                                        |
|          | 1,000       | 3                              | 10                 | 5                   | 13                                       |
|          | 10,000      | 5                              | 14                 | 13                  | 13                                       |
| KS-2     | 10          | 0                              | 0                  | 0                   | 7                                        |
|          | 100         | 0                              | 0                  | 0                   | 53                                       |
|          | 1,000       | 0                              | 0                  | 0                   | 47                                       |
|          | 10,000      | 0                              | 0                  | 1                   | 73                                       |

*Each chemical dissolved in methanol (200 µl) was loaded on a 5 mm paper disk.

Inhibition of mycelial growth was determined 5 days after incubation on PDA at 30°C.

0.5 ml of methanol dissolved with each chemical was loaded on a piece of sterile Kim-Wipe tissue (2 × 2 cm) and dried. Five seeds of radish in 3 replications were placed on the tissue for 3-5 days under normal day light.
The chemical structure of (A) KS-1 (2,4-diacetylphloroglucinol) and (B) KS-2 (2,4,6-trihydroxyacetophenone) isolated from the culture filtrate of *Pseudomonas brassicacearum* YC5480.

Discussion

The endophytic bacterial strain, YC5480 isolated from the root of *Artemisia* sp. with antifungal and phytotoxic activities was identified as *P. brassicacearum*. The species of *P. brassicacearum* was originally isolated from the rhizosphere of *Brassica napus* and *Arabidopsis thaliana* in France and some isolates of the species were also found in wheat field soils of two distinct geographic locations in Australia (Achouak et al., 2000; Ross et al., 2000). Some isolates had *in vitro* antagonism against the take-all fungus of wheat, *Gaeumannomyces graminis* var. tritici and suppressed take-all in pot trials (Ross et al., 2000). This indicates that isolates of *P. brassicacearum* have potential for use as biological control agent of plant pathogens (Fromin et al., 2001; Ross et al., 2000). However, no phytotoxic effect was reported on these strains of *P. brassicacearum* in spite of its importance as the biological control agent. The inhibition of seed germination and shoot growth of radish and other crops by the strain of *P. brassicacearum* has been first reported in this study. One strain 520-1 of the species was found to be pathogenic to tomato seedlings with chlorotic leaflets, vascular browning and necrotic lesions (Sikorski et al., 2001). It was frequently found that some bacterial endophytic isolates from healthy plants inhibited the growth of plant seedlings in inoculation tests, possibly through the production of certain metabolites (van Peer et al., 1990; Rosenblueth and Martinez-Romero, 2006).

The inhibition of seed germination and shoot growth of all tested crops in the culture filtrate of *P. brassicacearum* YC5480 regardless of monocot or dicot plants indicates that the inhibitory substance may be secreted into the culture media. Some strains of *Pseudomonas fluorescens* are known to suppress root and shoot growth of a grassy weed and bentgrass by producing peptides, fatty acid esters, a lipopolysaccharide matrix and indole-3-acetic acid (Gurusiddaiah et al., 1994; Suzuki et al., 2003). Recently, seed coating of barley and wheat seeds with *Pseudomonas chlororaphis* 06 at the same level as that in field soil was also found to inhibit seed germination by production of phenazine (Kang et al., 2007). The inhibition of radish seed germination by the strain of *P. brassicacearum* YC5480 was higher in the culture media, 1/2 TSB and CDB than other tested media which contained much more nutrients than the former two media. This may be due to the production of more amounts of active compounds KS-1 and KS-2 in these two media with relatively less nutrients than other rich media. The production of antifungal compounds and biocontrol activity of the bacteria is affected by cultivation conditions specially culture media (Bae et al., 2007). An important step in the development of a biological control agent is to determine the culture media for the efficient production of high yields of viable cells without accumulation of toxic compounds to microbes and plant growth. In addition, the cultivation conditions can be adjusted to optimize best cell activity and minimum production of inhibitory compounds (Slininger and Shea-Andersh, 2005).

Two antifungal and phytotoxic substances KS-1 (DAPG) and KS-2 (THA) were produced simultaneously in the culture broth of *P. brassicacearum* YC5480. The metabolites have also been observed to accumulate in liquid cultures of other pseudomonad strains and THA is likely a precursor or co-product during the biosynthesis of DAPG (Harrison et al., 1993; Shanahan et al., 1993; Slininger and Shea-Andersh, 2005). Both substances purified in this study inhibited the germination of radish seeds as in other studies (Reddi et al., 1969; Slininger and Shea-Andersh, 2005).
The substance DAPG inhibited mycelial growth of fungal pathogens at low concentrations. It is well recognized as a broad-spectrum antifungal substance produced by fluorescent pseudomonads that can control soilborne pathogens in the rhizosphere (Landa et al., 2003; Shanahan et al., 1992; Weller, 2007).

In this study, the strain of *P. brassicacearum* produced a phytotoxic substance THA as well as an antifungal substance DAPG during the cultivation. These results suggest that optimum cultivation conditions of this strain need to be determined to minimize the production of phytotoxic substances and maximize that of viable cell mass for the development as a biological control agent for plant diseases.

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