J. Microbiol. Biotechnol. (2000), 10(4), 435–440

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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Screening and Identification of an Antifungal *Pseudomonas* sp. That Suppresses Balloon Flower Root Rot Caused by *Rhizoctonia solani*

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Received: November 25, 1999
Accepted: June 21, 2000

Abstract A pathogenic fungus causing balloon flower root rot (*Platycodon grandiflorum*) was isolated from naturally infected roots. The microbial characteristics of the isolated microorganism were similar to those of *Rhizoctonia solani*. About 500 bacterial species from field soils were screened for a biological agent against the above-mentioned putative pathogen, and several bacteria with the antifungal activity were isolated. Among them, the isolated JS2 was identified as *Pseudomonas aeruginosa*. This strain showed a broad spectrum of antifungal activity potentially. When the antifungal substance was purified from a broth culture of JS2, it was identified as 2,4-diacetylphloroglucinol (Phl).

Key words: Balloon flower root rot, *Platycodon grandiflorum*, *Rhizoctonia solani*. *Pseudomonas aeruginosa*, 2,4-diacetylphloroglucinol (Phl)

Balloon flower (*Platycodon grandiflorum*) is widely cultivated and used in East Asia as a remedy for asthma and also consumed as a vegetable [17]. The plant is prone to several known diseases such as stem rot, Anthracnose, and *Fusarium* wilt [16]. However, root rot, which causes serious problems for the cultivation of balloon flower, had not been described until recently, and its cause has not yet been scientifically investigated. Balloon flower root rot is thought to be caused by some kind of soil sickness that develops because of continuous cropping, and mainly affects on the 3- to 5-year-old roots (personal communication). In fact, the predominant symptom of soil sickness is root rot, and *Rhizoctonia* is the most common pathogen to cause it.

Occasional root rot outbreaks caused by *Rhizoctonia solani* are difficult to control with chemicals. Furthermore, medicinal crops have traditionally been cultivated without the use of chemicals. Thus, a new strategy to control the pathogen causing balloon flower root rot is urgently needed. The most desirable method of control would be the use of a biological agent, and attempts to develop biological controls of plant pathogens have attracted the interest of an increasing number of scientists over the last 25 to 30 years [4]. Biocontrol mechanisms are generally classified based on their mode of action: such as competition for nutrition, parasitism/predation, and antibiosis [1, 8, 14, 19, 24]. Antibiosis is regarded as the single major important biological control mechanism. It is based on the inhibition of undesirable organisms by antibiotics and, consequently, competing microorganisms gain an advantage in the competition for nutrients and spaces within an ecological niche [20].

The present paper reports on the isolation of the fungus from naturally infected roots that causes balloon flower root rot. Antifungal bacteria were then screened for a biocontrol agent. A bacterium found to be suitable was identified and characterized, and the structure of its antifungal substance was identified using spectrometric techniques such as UV, FT-IR, FAB-MS, EI-MS, and FT-NMR spectroscopy.

MATERIALS AND METHODS

Microorganisms, Culture, and Growth Conditions

The phytopathogenic fungi *Pythium ultimum*, *Phytophthora capsici*, and *Fusarium oxysporum*, and the bacteria *Pseudomonas putida* and *Escherichia coli* were kindly provided by the Laboratory of Phytopathology, Kyungnam Agricultural Research and Extension Services, Chinju, Korea. The plant pathogenic fungi were maintained on potato dextrose agar (PDA), *Escherichia coli* was on Luria-Bertani agar, and *Pseudomonas* spp. were on Pseudomanas agar F (PAF).

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Isolation of Phytopathogens

Afflicted balloon flower roots were collected from two fields, which continuously cultivate balloon flower, at Aneui-Myun, Hamyang-Kun and Daepyung-Myun, Chinju, Korea. Samples were prepared from affected tissue as described previously [22, 23], maintained on PDA supplemented with streptomycin (100 mg/l), and incubated at 28°C for 3 days. The isolated fungi were stored at 5°C for storage and further study.

Pathogenicity Test of *R. solani*

For inoculation in the laboratory, a sclerotium sample of an isolated fungus grown on PDA at 27°C for 9 days was placed on the top part of the root of a 3-year-old intact balloon flower. The inoculated root was then left in a green house controlled at 25°C and 95% relative humidity. For control samples, uninoculated balloon flower roots were observed under the same conditions as above.

Screening of Bacteria for Antifungal Activity

Ten-grams of every field soil sample collected from 50 regions in Kyongnam Province, Korea was mixed with 100 ml of 0.1% Tween20 in distilled water (v/v) and shaken at 28°C for 30 min. The suspensions were then serially diluted and mixed with liquid phase PDA cooled to 45°C. The solidified plates were incubated at 25°C for 2 days, and single colonies were selected on the basis of their form and color. An *in vitro* bioassay to test antagonistic properties against the isolate of *R. solani* was conducted on the bacterial colonies, as previously described [3]. Selected bacteria were further tested for antagonistic properties against other phytopathogenic fungi including *Pythium ultimum, Phytophthora capsici, and Fusarium oxysporum.*

Taxonomic Studies

The morphological and microbiological characteristics of the pathogenic fungus were identified under a microscope. Sclerotia obtained from 10-day-old PDA cultures and stained with cotton blue in lactophenol were also examined. The bacterial strain isolated with a biologically active agent towards the fungal control was isolated and identified with the help of *Bergey's Manual* [10], MIDI™ (version 3.6 aerobic library:microbial ID, Inc., Netwark, Del. U.S.A.), VITEK™ (bioMetrieux sa, Marcy-l'Etoile, France), and Biolog™ (Biolog Inc., Hayward, CA, U.S.A.) [12].

Bioassay for the Biocontrol Activity of JS2

The *in vivo* assay of root rot suppression was conducted by a slightly modified method of that previously published [25]. Bottles of 2.5 cm (D) ×16.5 cm (L) dimensions were filled half-way with vermiculite (Fig. 3A). An inoculum made from 3 g of sterile soil and 0.1 g of *R. solani* sclerotium was spread on top of the layer of vermiculite and then overlaid with about 10 g of sterile soil. Balloon flower seeds were then sown at a depth of 1.5 cm into the soil. Three seeds were planted per bottle, and three replicates were made per treatment. Ten milliliters of tap water and the JS2 suspension culture (2×10^8 cells/ml) were added to each bottle, and the bottles were placed in a growth chamber kept at a relative humidity of 98%, temperature of 25°C, and 12 h photoperiod. Seeded bottles that received the same treatment except the bacterial inoculum and bottles that received only the seeds served as the control.

Purification and Structural Analysis of Antifungal Substance

The supernatant of broth cultured JS2 was extracted with an equal volume of chloroform, and the extract was concentrated. Subsequently, the chloroform fraction was purified by column chromatography with silicagel and preparative TLC plate. At each step of the purification, the fractions were tested for biological activity against *R. solani* by a paper disk assay procedure [9, 11]. Finally, a HPLC was performed using an HP1100 HPLC apparatus (Hewlett Packard, U.S.A.) and reverse phase column (ODS Hypersil 5 µm, C_{18} 200× 4.6 mm, Hewlett Packard, U.S.A.) to confirm its purity. The structure of the purified antifungal material was then examined using an EI-MS (VG70-VSEQ spectrometer, VG ANALYTICAL, U.K.), NMR (BRUKER, Germany), FT-IR (IFS, BRUKER, Germany), and UV spectroscopy (HP8452A, Hewlett Packard, U.S.A.) [18].

Determination of Antifungal Potency

The purified antifungal compound was dissolved in ethanol and added to the growth media at concentrations ranging from 0 to 1.021 µg/ml. For controls, the medium contained only 0.1% (v/v) ethanol without antifungal compound. A 6 mm plug of a 3-week-old PDA culture of one of the various fungi tested was placed in the center of each malt agar plate, and the plates were then incubated at 27°C in the dark. After 7 days, the mycelial growth was measured [13].

RESULTS

Identification of the Pathogen of Balloon Flower Root Rot

The affected balloon flower roots were collected in late autumn from several fields near Chinju and Hamyang, Korea. The isolated fungus from the diseased roots reproduced naturally-occurring typical symptoms in an artificial inoculation test performed in the laboratory (data not shown). After two weeks, the shoot languished and the root started to rot gradually, having a dark brown appearance. Thus, *R. solani* was shown to have a very potent pathogenicity towards the balloon flower plant. The fungus existed as a sterile mycelium with a pale brown

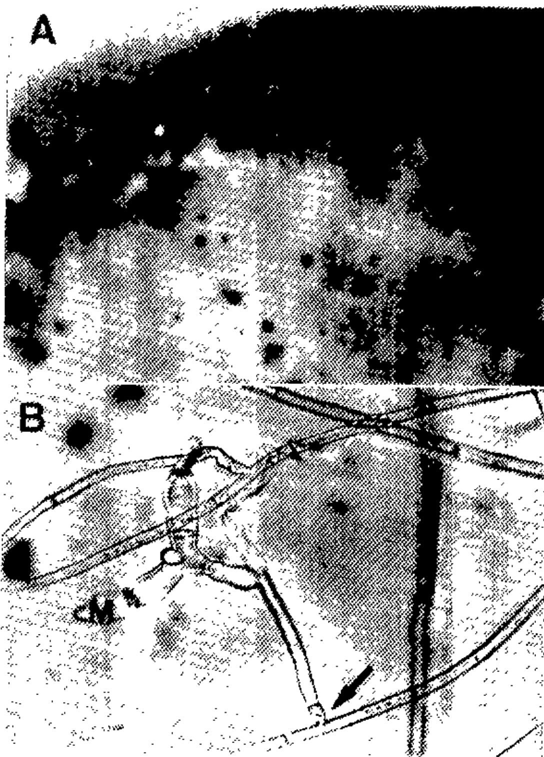

Fig. 1. Structural characteristics of isolated *Rhizoctonia solani*. A, surface of sclerotium B, microscopic structures of hyphae (M, monillioid cell; →, constricted hyphae)

color while it was still young, and then turned to a more brown color as it aged. The mycelium produced branches that grew at approximate right angles to the main hyphae. These hyphae were constricted at the junction, and cross wall occurred near the junction. The fungus produced sclerotia-like turfs of ovate, and black cells and monillioid cells when grown on PDA for 10 days at 28°C (Fig. 1B). The structures on the surface and in the core of the sclerotium were not histologically distinct (Fig. 1A). The optimal temperature for the growth of the isolated fungus on PDA was 28 to 29°C. The fungus grew about 2.5 cm a day on PDA at 29°C. The mycelial structures and physiological properties of the isolated fungus thus resembled those of a typical *R. solani*.

Screening for Bacteria with Antibiotic Activity against Fungal Phytopathogens

Nearly 500 bacterial isolates from field soils were screened for their color and morphology. About 30% exhibited antifungal activity against *R. solani*. Several of these antifungal bacteria were selected (Table 1), as well as two strains with a broad spectrum of antifungal activity in *in vitro* tests, designated as JS2 and JS4. These bacteria exhibited a strong antifungal effect on several major phytopathogens including *Pythium ultimum*, *Phytophthora capsici*, and *Fusarium oxysporum* as well as *R. solani* (Fig. 2). The JS2 was chosen for further in-depth studies.

Table 1. *In vitro* inhibitory activity against root pathogens by *Pseudomonas* sp isolates

Isolate	*Rhizoctonia solani*	*Fusarium oxysporum*	*Pythium ultimum*	*Phytophthora capsici*
Control	N/A	N/A	N/A	N/A
JS1	5	5	2	4
JS2	7	9	5	10
JS3	5	4	1	4
JS4	9	8	4	8
JS5	5	6	2	5
JS6	6	4	0	4
JS7	5	6	1	6
JS8	6	4	3	4
JS9	5	5	1	5
JS10	5	4	2	4
PF1	5	5	3	5
PF2	6	6	0	4

*Abbreviated as follows; N/A, not assayed.
**Antifungal activity was measured based on the width of the clear zone between the fungal pathogen and the bacterial colony.

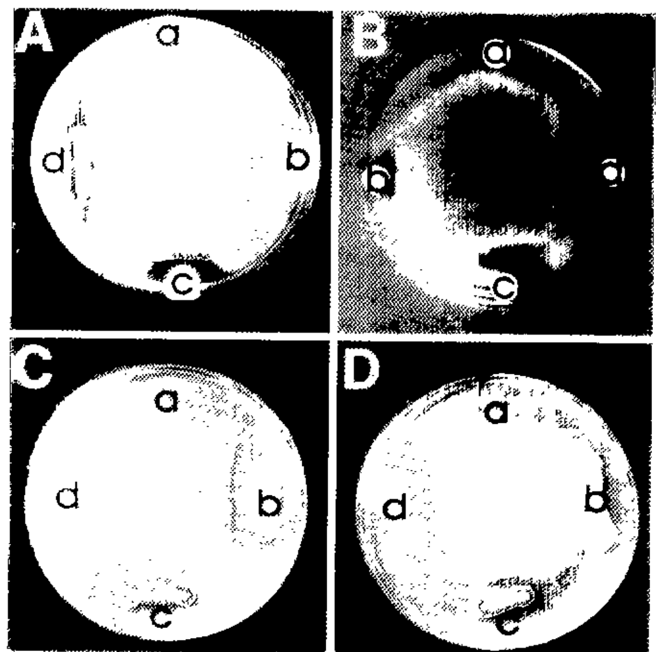

Fig. 2. Antagonistic activity of *Pseudomonas* sp. against the pathogen causing balloon flower root rot (B) and three other major plant pathogens: *Pythium ultimum* (A), *Phytophthora capsici* (C), *Fusarium oxysporum* (D), and (a) *E. coli*, (b) *Pseudomonas putida*, (c) JS4, and (d) JS2.

Identification of Isolate JS2 as *Peudomonas aeruginosa*

The JS2 exhibited a gram-negative and oxidase-positive reaction and was strictly aerobic. It produced a diffusible greenish-yellow fluorescing pigment on a PAF medium (Difco), however, it did not produce pyocyanine on a PAP medium (Difco). Based on a 49% similarity, the Biolog

Table 2. Comparison of bacteriological characteristics of JS2 with descriptions of *Pseudomonas aeruginosa*.

Character	Isolate JS2	*Peudomonas aeruginosa*
Gram stain	-	-
Aerobic growth	+	+
Fluorescent pigment	+	d
Oxidase reaction	+	+
Growth at 41°C	-	+
Pyocyanin	-	d
Utilization of:		
L-arabinose	-	-
D-sorbitol	-	-
Trehalose	+	-
α-D-glucose	+	+
m-inositol	+	-
D-manose	+	+
D-manitol	+	+
Psicose	+	+
α-lactose	-	-

-, no growth; +, normal growth, d, strain-dependent

system, which exploits the bacterial utilization patterns of various carbon sources, identified the JS2 as *Pseudomonas aeruginosa* (Table 2). In the VITEK system, the JS2 was identified as *P. aeruginosa* with an 81% similarity (data not shown). According to the above results, it was concluded that the JS2 was probably *Pseudomonas aeruginosa*. However, the MIDI system, which compares the fatty acid membranes compositions, was unable to identify the JS2.

Antagonistic Activity of the JS2 in *In Vivo* Tests

The JS2 significantly suppressed balloon flower root rot in the bottle assay. The seedlings potted in soil, which were not treated with the JS2 but inoculated with *R. solani*, germinated completely, yet languished and died soon after (Fig. 3-B1). The seedlings grown in soil treated with JS2 and *R. solani* (Fig. 3-B2) remained healthy and grew more quickly than those in the non-treated soil (Fig. 3-B3).

Isolation and Identification of Antifungal Compound

About 35 mg of the pure antifungal substance was isolated using column chromatography and preparative TLC from a 100 liters JS2 culture. The compound showing a single peak on HPLC chromatogram was subjected to ^{1}H-NMR, ^{13}C-NMR, infrared, UV, and EI mass spectroscopies. The spectroscopic data of this compound were as follows: IR(KBr) 3625, 3015, 2924, 1737, cm^{-1}; UV(MeOH) λmax 270, 330 nm; ^{1}H-NMR (500 MHz) δ 2.64(s,6H), 6.23(s, 1H); ^{13}C-NMR(125 MHz) δ 33.4, 96.1, 105.3, 170.6, 173.0, 205.6; EI-Mass 210(M$^+$, 87), 195(100), 177(86), 149(17), 67(35).

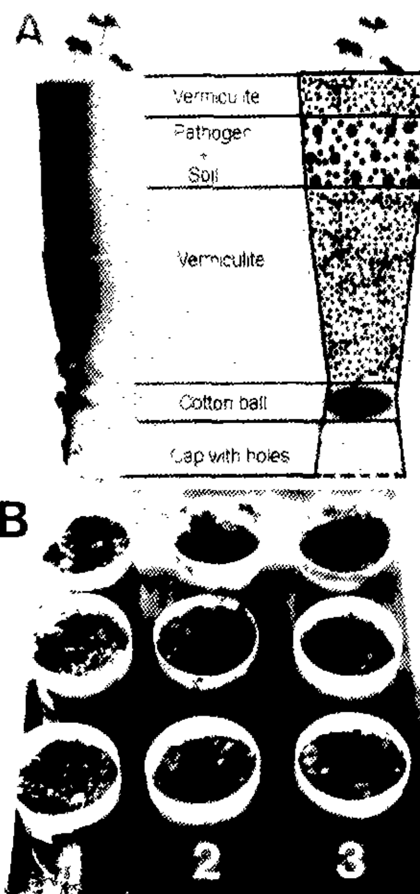

Fig. 3. Suppressive activity of strain JS2 against *Rhizoctonia solani* in bottle tests.
A, bottle for test; B1, pathogen only, B2, pathogen + strain JS2; B3 untreated control.

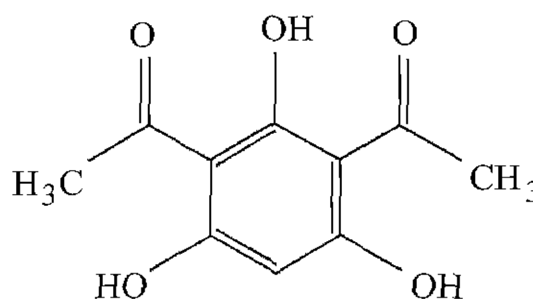

Fig. 4. Structural formula of the 2,4-diacetylphloroglucinol.

These data were in agreement with previously published data [7]. It was, therefore, concluded that the antifungal substance derived from JS2 was 2,4-diacetylphloroglucinol (Phl) (Fig. 4).

Antifungal Activity of Purified Phl

The antifungal compound exhibited a moderate antibiotic activity against soilborn fungal phytopathogens (Table 3). The I_{50} of *Fusarium oxysporum* was 17 μg/ml and I_{100} was

Table 3. Antifungal activity of Phl against several phytopathogenic fungi.

Fungus Tested	MICs (μg/ml)*	
	I_{50}	I_{100}
Fusarium oxysporum	17	129
Phytium ultimum	66	130
Rhizoctonia solani	66	129
Phytophthora capsici	35	120

*MICs are defined as the minimal concentration of Phl causing 50% (I_{50}) or total (I_{100}) inhibition of fungal growth on malt agar after 7 days.

129 μg/ml, respectively. *Phytium ultimum* and *R. solani* had similar sensitivities. These results are in agreement with previous report [13].

DISCUSSION

Generally, balloon flower root rot occurs concomitantly with stem rot, and it is still unclear which part of the plant first exhibits the symptoms of infection. In the early stage, the interface between the stem and the root is infected first, and most of the upper part of the root is infected with the pathogen early on. Once the pathogen has infected the balloon flower root, the shoot languishes, and it eventually rots off. This disease proceeds during summer to early winter until the whole root is finally rotten. The *R. solani* infection of balloon flower roots in test pots produced symptoms nearly identical to those of naturally-occurring balloon flower root rot in the field. These observations, therefore, strongly suggest that *R. solani* plays a major role during the occurrence of balloon flower root rot in the field.

Soil was collected from the field after harvest in late November to search for a biological agent that could control *R. solani*. This strategy was based on the principle that effective antagonists could generally be found in local soils and, even more likely, be associated with local crop plants. Among the bacteria isolated as biological agents, many of them belonged to the genus *Bacillus*, however, most of them exhibited a relatively lower antifungal activity than *Pseudomonas* spp. Also, it has generally been assumed that *Pseudomonas* spp. were efficiently colonized in the rhizosphere [15]. The JS2 exhibited a broad antifungal spectrum, and prevented the growth of major phytopathogenic fungi such as *Phytophthora capsici*, *Fusarium oxysporum*, *Pythium ultimum*, and the isolated *R. solani*. Many bacteria isolated as potential biocontrol agents exhibit pathogen-specific antifungal activity, however, a broad antifungal spectrum is generally more desirable to crop growers. The bioassay results, showing that the seedlings grown in soil treated with JS2 and *R. solani* grew more quickly than those of control, suggested that JS2 has not only antagonistic activity, but also plant growth promoting activity.

The JS2 was identified as *P. aeruginosa*. Previously, a *P. aeruginosa* has been isolated as a biological species that produces the antifungal substance, phenazine [7]. However, to our knowledge, this is the first report on a *P. aeruginosa* producing 2,4-diacetylphloroglucinol (Phl). The antifungal substance produced by the JS2 strain was identified by spectroscopy. The spectrograms obtained from UV, EI-MS, and ^{13}C-NMR were consistent with Phl [6]. Phl was first isolated as a phytotoxin [21] from *Pseudomonas fluorescens*, and it was later isolated as a major antibiotic for the use of the biocontrol of bacteria. Three Phl derivatives with antifungal activity against *Cladosporium herbarum* have been isolated from *Helichrysum decumbens*. In addition, Cronin *et al.* [5] recently reported that Phl exhibited anti-nematode activity both *in vitro* and *in vivo*. A phloroglucinol derivative with antifungal activity has also been found in a plant [2], and this derivative is mainly located in the surface layers of plant tissue and, thus, may play a role as a first line of defense against fungal invasion. This finding offers a possibility of creating transgenic plants with resistance to phytopathogens. Studies on soil-inhabiting antifungal agents are promising a great future in biocontrol of environmental pollution, health, and labor needs.

Acknowledgments

This research was funded by the Ministry of Agriculture and Forestry-Special Grants Research Program and by the Brain Korea 21 Project in part.

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