

Control Efficacy of *Streptomyces* sp. A501 against Ginseng Damping-off and Its Antifungal Substance

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Abstract Ginseng damping-off, caused by the fungal pathogens *Rhizoctonia solani* and *Pythium* sp., is a critical disease in ginseng seedling. In a continuing effort to find microorganisms with the potential of acting as a biocontrol agent against *Rhizoctonia* damping-off, we found that a *Streptomyces* sp. A501 showed significant antifungal activity against *Rhizoctonia solani*. In field experiment to test the efficacy of *Streptomyces* sp. A501 in controlling ginseng damping-off, the incidence of damping-off disease was meaningfully reduced when ginseng seeds were soaked in the culture broth of *Streptomyces* sp. A501 before sowing. To perform characterization of the antifungal compound, we isolated it from the culture broth of strain A501 through Diaion HP-20 and silica gel column chromatographies and preparative high-performance liquid chromatography. The structure of the antifungal compound was assigned as fungichromin by spectroscopic methods, mainly nuclear magnetic resonance and electrospray ionization-mass analysis.

Keywords Fungichromin, Ginseng damping-off, *Rhizoctonia solani*, *Streptomyces* sp. A501

Ginseng is a highly valued medicinal plant, which is cultivated in various regions, especially in Korea. The biological and pharmacological properties of ginseng are related to the presence of saponins, namely ginsenosides [1-5]. Damping-off disease is one of the most important diseases of ginseng and of many herb crops. It is caused by several fungi, such as *Pythium* sp. and *Rhizoctonia solani*. In many previous studies on microorganisms with the potential to act as

biocontrol agents against damping-off disease, several actinomycetes have been shown to have the ability to inhibit the growth of the mycelium of fungi [6]. Actinomycetes, including genus *Streptomyces*, have the ability to produce cell-wall-degrading enzymes or other toxic substances including antifungal substances, which play a major role in the biological properties of *Streptomyces* [7-9]. These results suggest that *Streptomyces* could be a potent biocontrol agent [10, 11].

In a previous study, *Streptomyces* sp. A501 has been reported to have potent antifungal activity against *R. solani* with *in vitro* antagonistic effect on ginseng damping-off [12]. The strain A501 was most closely related to members of the genus *Streptomyces* by phylogenetic analysis on the basis of 16S rRNA gene sequence and exhibited high similarity values of 100% with *Streptomyces murinus* group (*S. costaricanus*, *S. graminearus*, *S. griseofuscus*, and *S. murinus*) [12]. In this study, the control efficacy of *Streptomyces* sp. A501 against ginseng damping-off disease in the field was investigated and an antifungal substance was successfully isolated from the culture broth of *Streptomyces* sp. A501 with the help of chromatographic methods and characterized by spectroscopic methods, mainly nuclear magnetic resonance (NMR) and electrospray ionization (ESI) mass spectrometry.

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Fermentation of strain A501. Strain A501 was maintained on modified Bennett's agar plates at 27°C for 5 days. The strain was pre-cultured in two 1-L Erlenmeyer flasks, each flask containing 200 mL GSS broth (glucose 20 g, soluble starch 10 g, meat extract 1 g, yeast extract 4 g, sodium chloride 2 g, potassium phosphate dibasic anhydrous 0.05 g, soybean flour 25 g/L, pH 7.0), for 2 days on a rotary shaker at 120 rpm at 27°C. The fermentation was scaled up to 3 L with the same medium as above and incubated under the same conditions as described above for 5 days.

Control efficacy against ginseng damping-off disease in the field. The control efficacy of *Streptomyces* sp. A501 against ginseng damping-off disease in the field was performed at the experimental field of the Chungnam National University, Yuseong, Korea, in 2016. Briefly, ginseng seeds were soaked in 100-fold diluted culture broth of *Streptomyces* sp. A501 for 30 min and dried in the shade. Each seed was sown at an interval of 3 × 3 cm with a sowing plate. The seeding area for each treatment group was 90 × 360 cm (three replicates per treatment group). Seeds soaked in distilled water for 30 min were used as control. Control efficacy is a control effect against naturally occurring damping-off disease without artificial treatment with specific pathogens.

The germination rate of the seeds and incidence of damping-off disease were investigated at 50 days after the seeds were sowed. The germination rate of the ginseng seeds was 75%, which was slightly higher than control (73%) (Fig. 1A). The incidence of damping-off disease was meaningfully reduced up to 44% when ginseng seeds were soaked in the culture broth of *Streptomyces* sp. A501 (Fig. 1B).

Extraction and isolation of antifungal substance.

Antifungal substance was isolated from the culture broth of *Streptomyces* sp. A501 by the antifungal activity-guided fractionation. The culture broth of *Streptomyces* sp. A501 was centrifuged at 6,000 rpm for 25 min to separate the supernatant and mycelium. The mycelium was extracted with acetone for 24 hr and concentrated *in vacuo*. Both the supernatant and acetone extract exhibited antifungal activity against *R. solani*. The supernatant and acetone extract were combined and subjected to Diaion HP-20 column chromatography (Mitsubishi Chemical Industries, Tokyo, Japan), eluted stepwise with MeOH-H₂O (0 : 100, 30 : 70, 50 : 50, 70 : 30, 100 : 0, v/v) and washed with acetone. Two fractions (70% aqueous MeOH and MeOH fractions) exhibited antifungal activity. An active fraction, 70% aqueous MeOH eluate, was concentrated under reduced pressure, and the residue was dissolved in water and partitioned between ethyl acetate and water. The ethyl acetate layer was evaporated under reduced pressure to give an ethyl acetate-soluble residue. Another active fraction, MeOH eluate, was evaporated under reduced pressure and combined with the ethyl acetate-soluble residue. The combined active residue was subjected to silica gel column chromatography, eluted with CHCl₃-MeOH (10 : 1, 5 : 1, 2 : 1, 1 : 1, v/v, stepwise) to give an active fraction (CHCl₃-MeOH = 5 : 1, v/v), followed by preparative high-performance liquid chromatography eluted with 26% aqueous acetonitrile at a flow rate of 3 mL/min to afford compound **1** (8.4 mg) (Fig. 2).

Structure determination of antifungal compound.

The structure of antifungal compound **1** was determined by ESI-mass measurement, one-dimensional ¹H and ¹³C

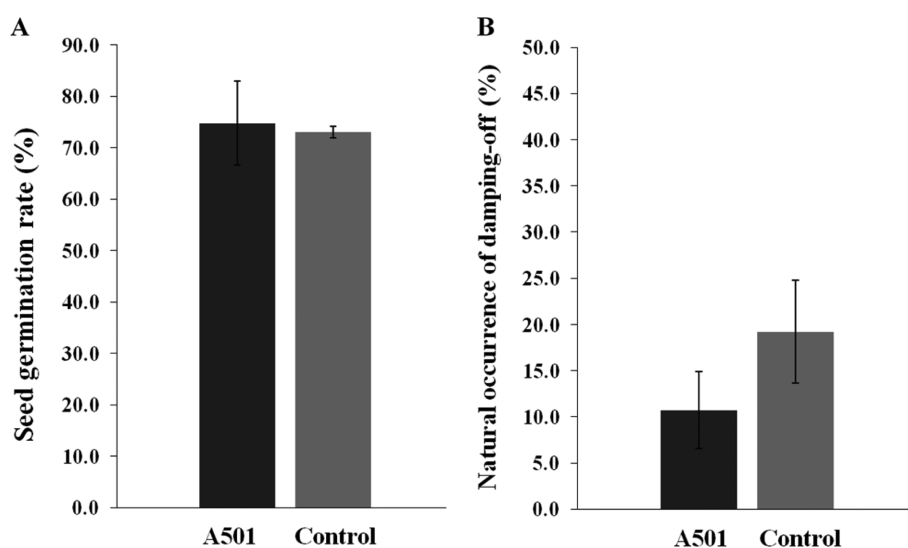


Fig. 1. Effects of *Streptomyces* sp. A501 on the germination rate of the ginseng seeds (A) and the incidence of ginseng damping-off (B). Ginseng seeds were soaked in 100-fold diluted culture broth of *Streptomyces* sp. A501 for 30 min and dried in the shade. Each seed was sown at an interval of 3 × 3 cm with a sowing plate. The seeding area per treatment was 90 × 360 cm (three replicates per treatment). Seeds soaked in distilled water for 30 min were used as control. Germination rate and natural incidence of ginseng damping-off were examined at 50 days after sowing.

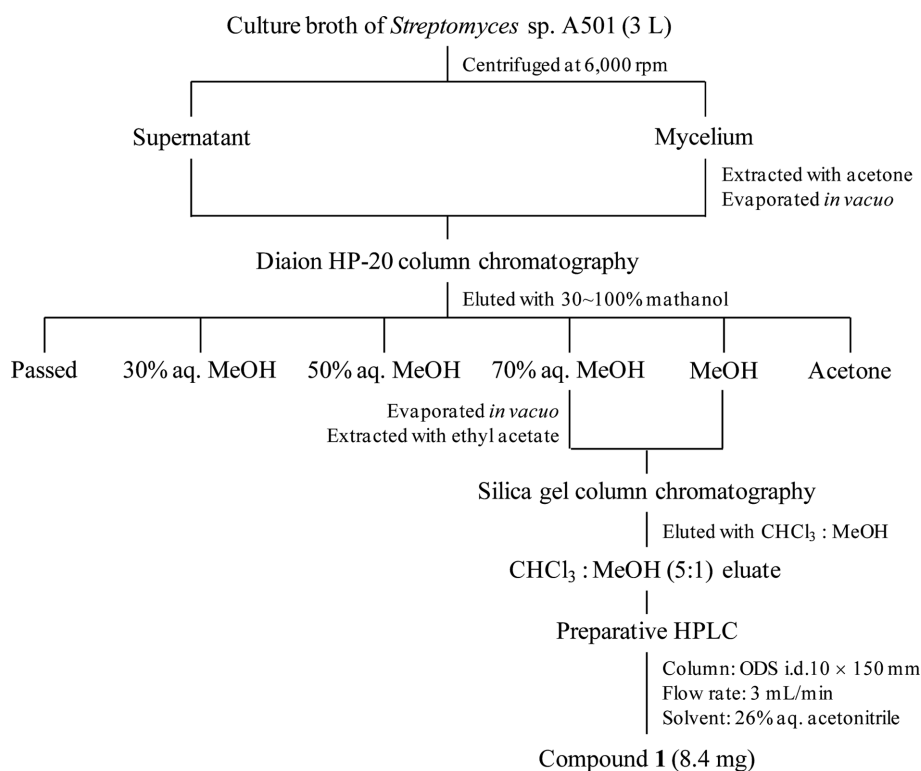


Fig. 2. Isolation and purification procedure of compound 1. HPLC, high-performance liquid chromatography.

NMR spectra, and two-dimensional ^1H - ^1H COSY, HMQC, and HMBC spectra.

ESI-mass was obtained on an Agilent Technologies 6410 Triple Quad LC/MS spectrometer (Agilent Technologies, Santa Clara, CA, USA) in positive and negative modes. The molecular weight of **1** was established as 670 by a quasi-molecular ion peaks at m/z 693 $[\text{M}+\text{Na}]^+$ in positive mode and at m/z 669 $[\text{M}-\text{H}]^-$ in negative mode. The UV spectrum suggested that **1** had a character of polyene compound with five conjugated double-bonds. To determine chemical structure, NMR spectra were obtained by using a JEOL 600 MHz FT-NMR spectrometer (JEOL, Tokyo, Japan) at 600 MHz for ^1H NMR spectrum and at 150 MHz for ^{13}C NMR spectrum in CD_3OD . Chemical shifts were given in ppm (δ) using tetramethylsilane as an internal standard. The ^1H NMR spectrum exhibited signals due to nine olefinic methines at δ 6.01–6.44, eleven oxygenated methines at δ 3.24–4.82, one methine at δ 2.54, 18 methylenes at δ 1.24–1.75, and three methyls at δ 0.89, 1.28, and 1.77. In the ^{13}C NMR spectrum, 35 carbon peaks were evident, including: a carbonyl group at δ 171.6, one sp^2 quaternary carbon at δ 137.2, nine sp^2 methine carbons at δ 127.7–134.1, eleven oxygenated methine carbons at δ 68.9–79.1, one methine carbon at δ 59.1, nine methylene carbons at δ 22.4–43.8, and three methyl carbons at δ 10.3, 13.1, and 16.6. The ^1H - ^1H COSY spectrum established three partial structures, as shown in Fig 3. Other cross-peaks in the COSY spectrum were seriously overlapped. Although the HMBC spectrum extended the partial structures,

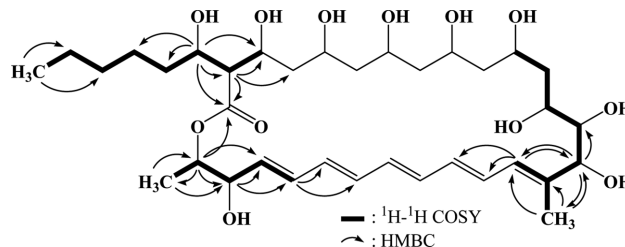


Fig. 3. Two-dimensional nuclear magnetic resonance data of compound 1 (fungichromin).

as shown in Fig. 3, the structure of compound **1** could not be determined because of serious overlapping of HMBC cross-peaks. However, the UV spectrum, suggesting a polyene moiety with five conjugated double-bonds, together with mass spectrometric data suggested that compound **1** was identical to an antibiotic fungichromin (Fig. 3) [13–15]. Finally, this conclusion was supported by ^1H and ^{13}C NMR spectral data, which were consistent with those of fungichromin [16].

Polyenes are a group of macrolide antibiotics that selectively damage a wide variety of fungi. Fungichromin, a polyene macrolide called pentamycin, was previously isolated from *Streptomyces pentaticus* as an antifungal antibiotic showing antifungal activity against various fungal pathogens. Fungichromin has already been approved for the topical treatment of bacterial and fungal vaginitis and administered as a vaginal tablet [17]. In this study, we found that

Streptomyces sp. A501 producing a polyene fungichromin was effective in controlling ginseng damping-off disease in the field test. Fungichromin is a major ingredient of the culture broth of *Streptomyces* sp. A501 and seems to play an important role in the growth inhibition of the damping-off pathogen *R. solani*. These results suggest that the *Streptomyces* sp. A501 could be used to control ginseng damping-off disease caused by *R. solani*.

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