

Antagonistic Effect of *Streptomyces* sp. BS062 against *Botrytis* Diseases

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Abstract The use of microorganisms and their secreted molecules to prevent plant diseases is considered an attractive alternative and way to supplement synthetic fungicides for the management of plant diseases. Strain BS062 was selected based on its ability to inhibit the mycelial growth of *Botrytis cinerea*, a major causal fungus of postharvest root rot of ginseng and strawberry gray mold disease. Strain BS062 was found to be closely related to *Streptomyces hygroscopicus* (99% similarity) on the basis of 16S ribosomal DNA sequence analysis. Postharvest root rot of ginseng and strawberry gray mold disease caused by *B. cinerea* were controlled up to 73.9% and 58%, respectively, upon treatment with culture broth of *Streptomyces* sp. BS062. These results suggest that strain BS062 may be a potential agent for controlling ginseng postharvest root rot and strawberry gray mold disease.

Keywords Biocontrol agent, *Botrytis cinerea*, Ginseng root rot, Gray mold disease, *Streptomyces* sp. BS062

Botrytis diseases such as *Botrytis* blight and gray-mold disease are caused by *Botrytis cinerea* Pers.: Fr., and are the most problematic diseases among greenhouse-grown crops and storage crops. *B. cinerea* is normally controlled using fungicides; however, the growing demand of consumers worldwide for a reduction in the use of fungicides as well as the emergence of chemical resistant pathogens has emphasized the need to find alternative methods for controlling gray mold disease [1, 2]. Biological control uses microorganisms and/or their secreted molecules to prevent disease and is considered an attractive alternative to fungicides for the management of plant disease. Importantly, biological control is not associated with any of the negative effects of chemical control, although relatively few antagonistic microbes have been commercialized as biocontrol agents due to problems such as inconsistent performance in the

field, lack of broad-spectrum disease suppression activity, and/or slower or less complete suppression compared to chemical alternatives [2-5].

Actinomycetes are recognized as potential sources of antibiotics and lytic enzymes with both medical and industrial value [5-7]. While only a few taxa (mainly *Streptomyces* spp.) have been studied as potential biocontrol agents against fungal phytopathogens, several fungicides based on antibiotics present in *Streptomyces* are commercially used in pest control. Jinglyngmycin, an antibiotic produced by *S. hygroscopicus* var. *jinglyngensis*, is widely used to control rice sheath blight caused by *Rhizoctonia solani* in China [5]. Likewise, Mycostop, a product containing mycelium and spore of *S. griseoviridis*, is used to control *Fusarium* wilt and *Botrytis* gray mold of vegetables or ornamentals in Finland [6].

In our screening program to identify microorganisms that have the potential to be useful as microbial fungicide for the control of *Botrytis* diseases, an effective bacterial strain BS062 was isolated from a soil sample. In this study, we tested the ability of strain BS062 to antagonize the growth of a wide variety of plant pathogenic fungi *in vitro*. In addition, the effect of BS062 culture broth against *Botrytis* diseases on strawberry and ginseng were evaluated *in vivo* and compared with a commercial fungicide.

Identification and fermentation of strain BS062.

Strain BS062 showing antagonistic effects against *B. cinerea* was isolated from a soil sample collected at Mt. Gyeryongsan, Korea. Strain BS062 was identified as belonging to genus *Streptomyces* on the basis of its structural and morphological

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characteristics (data not shown), and thus strain BS062 was designated as *Streptomyces* sp. BS062. 16s rDNA gene sequence analysis showed that strain BS062 was closely related to *Streptomyces hygrosopicus* (99% similarity, Genbank database accession No. AJ391820).

Strain BS062 was cultured in 100 mL of yeast-malt extract broth in a 500-mL Erlenmeyer flask on a rotary shaker at 150 rpm for 3 days at 28°C. A 5-mL aliquot of the culture broth was then transferred into 200 mL of yeast-starch broth (soluble starch 70 g, soybean meal 5 g, yeast extract 17 g, (NH₄)₂SO₄ 1 g, CaCO₃ 1 g in 1 L water) in a 1-L Erlenmeyer flask. A total of 20 flasks containing yeast-starch broth were inoculated with culture broth as described above and incubated for 6 days at 28°C on a rotary shaker with agitation at 150 rpm. After incubation, the resulting culture broth was centrifuged at 5,000 rpm for 20 min, and the supernatant was used for estimation of *in vitro* and *in vivo* biological activity.

Antifungal activity of *Streptomyces* sp. BS062. The ability of strain BS062 to inhibit the growth of several

Table 1. Antifungal activity of strain BS062 against plant pathogenic fungi

Microorganism	Antifungal activity ^a
<i>Alternaria mali</i>	++
<i>Alternaria porri</i>	++
<i>Botrytis cinerea</i>	+++
<i>Colletotrichum gloeosporioides</i>	+
<i>Cylindrocarpon destructans</i>	–
<i>Fulvia fulva</i>	++
<i>Fusarium oxysporum</i>	+
<i>Magnaporthe grisea</i>	++
<i>Phytophthora capsici</i>	–
<i>Pythium ultimum</i>	–
<i>Rhizoctonia solani</i>	–

^aThe antifungal activity of the strain BS062 against plant pathogenic fungi was assessed by dual culture technique. The levels of growth inhibition were determined by the differences between the diameters of the radial fungal growth of a control culture (γ_0) and the radial fungal growth of paired-cultures (γ) in the direction of strain BS062 as determined by the equation; $\Delta\gamma = \gamma_0 - \gamma$; +++, $\Delta\gamma \geq 20$ mm; ++, $\Delta\gamma \geq 10\sim 19$ mm; +, $\Delta\gamma \geq 5\sim 9$ mm; –, $\Delta\gamma < 5$ mm (no antifungal activity).

plant pathogenic fungi was estimated using the method described by El-Tarabily *et al.* [8], with minor modifications. Briefly, antifungal activity was evaluated based on its ability to inhibit mycelial growth (Table 1). *Streptomyces* sp. BS062 exhibited potent inhibitory activity against *B. cinerea*, *Alternaria mali*, *Alternaria porri*, *Fulvia fulva*, and *Magnaporthe grisea*, but had no activity against *Phytophthora capsici*, *Pythium ultimum*, *Cylindrocarpon destructans*, and *Rhizoctonia solani*. Notably, BS062 specifically and potently inhibited the growth of *B. cinerea* mycelia.

Suppression of ginseng root rot by culture broth of *Streptomyces* sp. BS062.

One-yr-old ginseng roots were surface-disinfected in a solution of 1% sodium hypochlorite for 20 min and rinsed with distilled water five times. The resulting ginseng roots were dipped in 10-fold diluted culture broth of strain BS062, diethofencarb + carbendazim (500 ppm) or distilled water. After drying at room temperature for 2 hr, the ginseng roots were inoculated by spraying with a conidial suspension (10⁶ conidia/mL) of *B. cinerea*. Roots inoculated by *B. cinerea* were kept for 30 days at 4°C. Disease severity (%) was measured by estimating the percentage of root rot at 15 and 30 days of inoculation. In addition, the numbers of ginseng plants exhibiting symptoms of infection were counted to determine efficacy with respect to controlling root rot. As shown in Table 2, treatment with culture filtrate significantly reduced the severity of ginseng root rot caused by *B. cinerea* (73.9% compared to controls) during storage at 4°C. In addition, the ability of strain BS062 to control root rot was comparable to that of the fungicide, a complex of diethofencarb and carbendazim. Effectiveness was positively correlated with the concentrations of culture broth (data not shown).

Suppression of strawberry gray mold disease by culture broth of *Streptomyces* sp. BS062.

In order to further evaluate the antagonistic activity of strain BS062 against *B. cinerea*, strawberry fruits were dipped in 10-fold diluted culture filtrate of strain BS062, diethofencarb (1,000-fold dilute, w/v), or sterile water before pathogen inoculation as described below. Briefly, *B. cinerea* spores were suspended in a solution of 20% tomato juice containing 0.1 M KH₂PO₄ to a concentration of 10⁶ conidia/mL. Aliquots of the *B.*

Table 2. Effect of *Streptomyces* sp. BS062 culture broth on the suppression of postharvest ginseng root rot by *Botrytis cinerea*

Treatment	Healthiness ^a (%)	Rot (%)		Stem after emergence (cm)	Control effect (%)
		RREG	REG		
Culture broth	77.5 ± 1.02	10.4 ± 1.90	12.2 ± 2.87	10.78 ± 2.94	73.9 ± 0.74
Diethofencarb + carbendazim	79.6 ± 4.46	7.6 ± 2.29	12.8 ± 2.35	15.49 ± 3.7	76.5 ± 4.61
Control	13.5 ± 1.96	47.8 ± 5.35	38.6 ± 6.39	12.58 ± 3.91	–

Experiments were done in triplicate with 100 ginseng roots for each group.

RREG, rotten rhizome and epidermis of ginseng root; REG, rotten epidermis of ginseng root.

^aHealthy ginseng root.

cinerea spore suspension were sprayed on strawberry fruits, which were then incubated in growth chambers at 20°C for 7 days. Strawberry fruits were individually rated for disease severity using a scale consisting of 0, 2, 4, 6, and 8; where 0, no symptoms; 2, $\leq 1/4$ of the fruit surface afflicted by gray mold disease; 4, $1/4\sim 1/2$ of the surface afflicted by gray mold disease; 6, $1/2\sim 3/4$ of the surface afflicted by gray mold disease; and 8, entire surface of the fruit afflicted by gray mold disease and covered with profuse aerial mycelium [9]. Efficacy was calculated by adding together the individual strawberry ratings and dividing by the total number of strawberry fruits. This bioassay was repeated three times.

After incubation for 7 days at 20°C, *B. cinerea*-inoculated strawberry fruits developed gray mold disease with formation of profuse mycelium on the fruit surface (Fig. 1C). In contrast, fruits treated with culture broth of BS062 ranged from healthy to the appearance of light symptoms (Fig. 1A). The efficacy of strain BS062 against strawberry gray mold disease was 58%. The average severity rating of strawberries infected with *B. cinerea* and treated with sterile water (control) and diethofencarb was 4.04 and 6.34, respectively, while that of strawberries treated with 10-fold diluted culture broth of strain BS062 was 2.5 (Fig. 1). Together, these results indicated that *Streptomyces* sp. BS062 was effective at controlling strawberry gray mold disease.

Inhibition of mycelia growth of *B. cinerea* by culture broth of *Streptomyces* sp. BS062. We next investigated the inhibitory effect of BS062 on mycelia growth of *B. cinerea* on potato dextrose agar medium according to the method described by Droby *et al.* [10]. Briefly, a 5-mm diameter plug of mycelia agar was obtained from the growing edge of 5-day-old cultures of *B. cinerea* and placed in the center of a plate containing potato dextrose agar medium and different concentrations (5-, 10-, 20-, 40-, and 80-fold dilutions) of culture broth, which was then filtered using a 0.2- μm filter (Millipore) before addition into autoclaved potato dextrose agar medium. The resulting diameters of *B. cinerea* growth were observed after incubation at 23°C for 3, 5, and 7 days, and results were recorded as the diameter of fungal growth (Fig. 2). The culture filtrate of strain BS062 potently inhibited the mycelial growth of *B. cinerea*. Specifically, after incubation for 3 or 5 days, the growth of *B. cinerea* was completely inhibited by the 5-fold dilution of the culture filtrate. While inhibition decreased slightly with increasing incubation time, we noted a positive correlation between the concentration of the culture filtrate and inhibition of mycelia growth. This result suggested that strain BS062 inhibited the *B. cinerea* mycelia growth through the production of extracellular antifungal antibiotics. Consistent with this possibility, production of secondary antifungal substances such as antibiotics has been reported in many species of *Streptomyces* [11-13]. In addition,

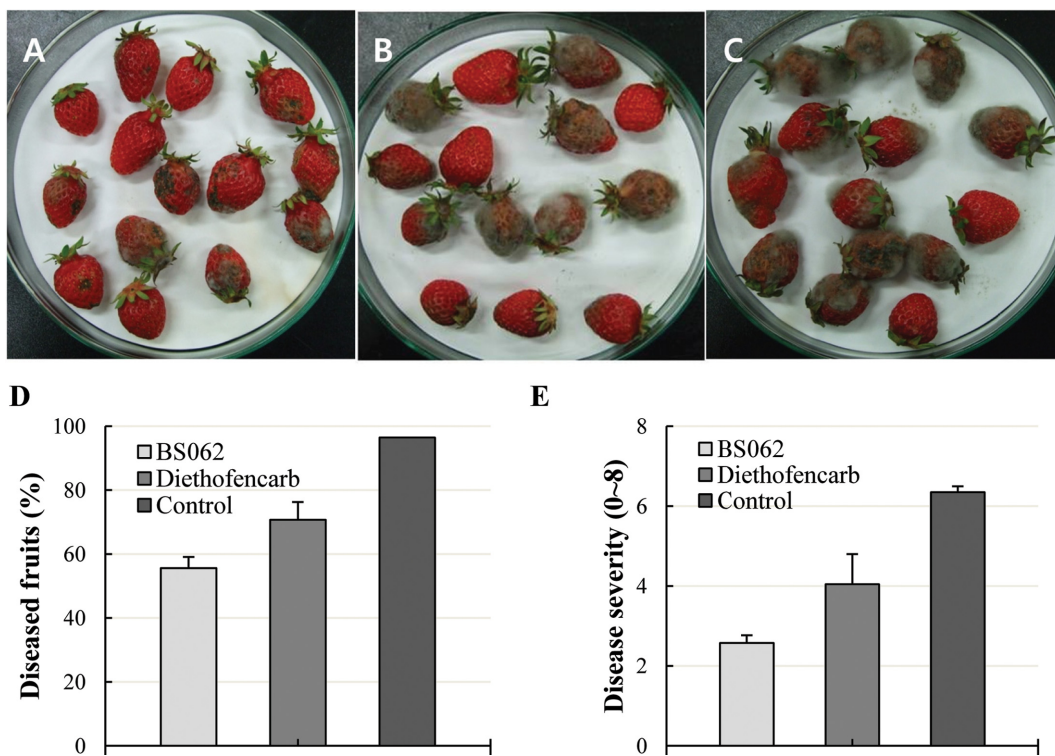


Fig. 1. The incidence and severity of *Botrytis* gray mold disease of strawberries in the absence or presence of *Streptomyces* sp. BS062. A, 10-fold dilution of the culture broth of *Streptomyces* sp. BS062; B, Diethofencarb (250 ppm); C, Control (sterilized water); D, Percent of diseased fruits; E, Disease severity. The statistical results for incidence and severity of each disease are expressed as the mean \pm standard error.

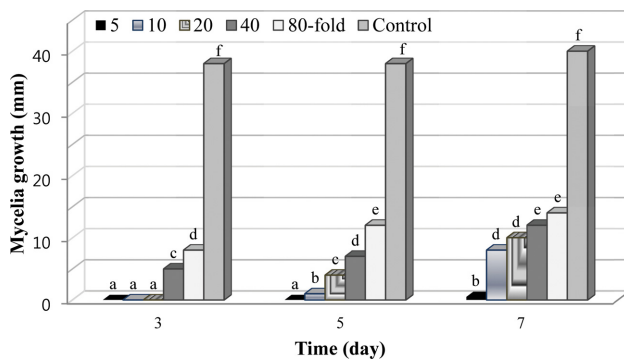


Fig. 2. Effect of various dilutions of *Streptomyces* sp. BS062 culture broth on *Botrytis cinerea* mycelia growth. Mycelial growth was monitored by measuring the colony diameter at 2-day intervals. Bars with different letters within each sampling interval are statistically different according to Duncan's multiple range test ($p < 0.05$).

according to a literature survey, *S. hygroscopicus* inhibits a broad range of fungal pathogens such as *Rhizoctonia solani*, *Pythium ultimum*, *Fusarium oxysporum*, and *Sclerotinia homeocarpa* [14, 15]; however, the antifungal spectrum of BS062 was different from that reported for *S. hygroscopicus*. Thus, the isolation of active substance from the culture broth of *Streptomyces* sp. BS062 is in progress.

Conclusion. In the course of screening for microorganisms with the potential to be used as biocontrol agents, *Streptomyces* sp. BS062 was identified as having potent antifungal activity against *Botrytis cinerea*, a major causal fungus of postharvest root rot of ginseng and strawberry gray mold disease. According to *in vivo* tests, *Streptomyces* sp. BS062 significantly reduced the severity of postharvest root rot of ginseng and gray mold disease of strawberry caused by *B. cinerea* with an efficacy of 74% and 58%, respectively. In addition, strain BS062 was found to be closely related to *S. hygroscopicus*, having a 99% similarity on the basis of 16S ribosomal DNA sequence analysis. However, despite this similarity, the antifungal spectrum of BS062 was distinct from what was previously reported for *S. hygroscopicus*. Although the specific antifungal substances produced by *Streptomyces* sp. BS062 remain to be identified, this strain has the potential to be a useful agent for the biological control of postharvest root rot in ginseng and strawberry gray mold disease.

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