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Research Article

Bacillus sp. BS061 Suppresses Powdery Mildew and Gray Mold

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Abstract The use of a microorganism, or its secretions, to prevent plant disease offers an attractive alternative or supplement to synthetic fungicides for the management of plant disease without the negative effects of chemical control mechanisms. During a screening for microorganisms with the potential to be used as microbial fungicides, *Bacillus* sp. BS061 was isolated from a plant leaf. The strain BS061 potently inhibited the mycelial growth of *Botrytis cinerea,* and significantly reduced disease incidence of powdery mildew in cucumber and strawberry. We also found that the culture filtrate of BS061 inhibited the mycelial growth of various plant pathogens.

Keywords *Bacillus* sp. BS061, Biocontrol effect, Gray mold, Powdery mildew

Biological control, the use of a microorganism (or its secretions) to prevent disease, offers an attractive alternative or supplement to fungicides for the management of plant disease, without any of the negative effects of chemical control [1, 2]. However, relatively few of these antagonistic microbes have been commercialized as biocontrol agents, due to problems such as inconsistent performance in the field, lack of a broad-spectrum disease suppression activity, or slower or less complete suppression when compared to chemical alternatives [3-5].

A number of Gram-positive and Gram-negative bacteria have been evaluated as biological control agents. Gram-negative bacteria, including *Pseudomonas cepacia* [6], *Pseudomonas syringae* [7], *Pantoea agglomerans* [8, 9], and *Serratia plymuthica* [10, 11] were reported to be effective against a number of diseases of different fruits. Several strains belonging to the genus *Bacillus*, in particular *B. subtilis* and the closely related *B. amyloliquefaciens*, were reported to be effective for the biological control of multiple plant diseases. Antibiotic production by these bacteria plays a major role in disease suppression [12]. Gram-positive bacteria, especially strains of *Bacillus subtilis*, produce diverse antibacterial and antifungal antibiotics, such as zwittermicin-A [12, 13], kanosamine [14], and lipopeptides of the surfactin, iturin, and fengycin families. Iturins and fengycins display strong antifungal activities, and inhibit the growth of a wide range of plant pathogens [15-17].

Diseases of gray mold and powdery mildew are very common plant diseases throughout various geographical regions, and occur under a variety of growing conditions. Powdery mildew is one of the most serious plant diseases worldwide, causing large yield losses in a number of crops [18]. The conventional chemical control of powdery mildew is through repeated foliar applications of a combination of protectant and systemic fungicides. But the intensive use of fungicides worldwide has resulted in an increased frequency of powdery mildew pathogens with reduced sensitivity to chemical fungicides. Gray mold disease, caused by *Botrytis cinerea* Pers.: Fr., is one of the most serious plant diseases affecting vegetables, ornamentals, and fruit crops produced in commercial greenhouses and fields all over the world [19, 20]. The control of *B. cinerea* is normally carried out by the application of fungicides. However, the growing demand of consumers worldwide for a reduction in the use of fungicides, as well as the appearance of pathogens resistant to chemical compounds, has emphasized the need to find alternative methods for the control of diseases caused by gray mold [21].

In our screening for microorganisms with the potential to be used as microbial fungicides for the simultaneous control of gray mold and powdery mildew, an effective

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bacterial strain of the *Bacillus* species, BS061, was isolated from the leaf of a plant. In this study, its ability to antagonize the growth of a wide variety of plant pathogenic fungi *in vitro* was tested, and the control effects of cell and cell-free culture broth against gray mold and powdery mildew were investigated *in vivo*.

MATERIALS AND METHODS

Cultivation. A loopful of *Bacillus* sp. BS061, which was maintained on a Mueller Hinton (MH) agar plate, was transferred into a 500 mL Erlenmeyer flask containing 200 mL of MH broth. The flasks were incubated on a rotary shaker at 200 rpm/min for 12 hr at 28°C. One milliliter of this fresh culture was inoculated into 500 mL Erlenmeyer flasks containing the same medium as above. The flasks were incubated under the same conditions as described earlier for 96 hr. Cells were then removed by centrifugation at 8,000 rpm for 30 min and subsequently the supernatant was filtered through a 0.2 μm polycarbonate membrane filter.

Control effect against powdery mildew in greenhouse. Cucumber plants were grown in the greenhouse until the 1~2 leaf stage, after which they were treated with a chemical fungicide and a culture filtrate of *Bacillus* sp. BS061. Treatments were made two times, with an interval of a week, starting immediately after the appearance of the first symptoms of powdery mildew on the foliage, which was naturally infected with the powdery mildew fungus. Fenarimol and triflumizole were applied as positive controls. Disease severity was rated on five leaves (leaves 2~6) per cucumber plant 7 days after the second treatment. Ten plants were used in each treatment, with 5 replicates. The incidence of powdery mildew was visually evaluated on individual leaves and ranked as a percentage of the infected area using a 0~4 index, where 0 = no symptoms, 1 = 0.1~5%, 2 = 5.1~20%, 3 = 20.1~40%, and 4 = 40.1~100%. In strawberry plant experiments, 10 plants were placed per meter ridge and approximately 50 plants were grown in a plot with three replicates. The disease incidence of strawberry was evaluated based on the number of infected fruits.

Control effects against gray mold in seedlings. The seedling plants were transplanted and cultivated under glasshouse conditions for 4 wk. To evaluate the antagonistic effect of *Bacillus* sp. BS061 against *B. cinerea*, the seedlings were sprayed with BS061 culture filtrate at 24 hr before inoculation of the pathogen. Each treatment contained 25 seedlings with three replicates. The pathogen, *B. cinerea*, was grown to sporulation on potato dextrose agar at 20°C and prepared by harvesting conidia from 15 day-cultures in sterile water. After removing mycelial debris by filtering through three layers of cheesecloth, the conidia were resuspended in a 20% tomato juice solution supplemented with 0.1 M KH_2PO_4 to a concentration of 4×10^6 conidia/mL. The conidial density of the suspension was determined using a hemocytometer. The disease incidence index of gray mold on the seedling plants was defined through the above method.

Antifungal activity. The antifungal activity of the culture filtrate was evaluated by a classical antibiotic test using the agar diffusion method in Petri dishes. Ten microliter aliquots of culture filtrate were dispensed in wells (performed with a sterile cork borer, diameter 6 mm) of an agar plate pre-seeded with various phytopathogenic fungi: *Botrytis cinerea*, *Magnaporthe grisea*, *Fusarium oxysporum*, *Fulvia fulva*, *Collectotrichum gloeosporiodes*, *Alternaria mali*, *A. panax*, *Phytophthora capsici*, *Pythium* sp., *Cylindrocarpon destructans*, *Rhizoctonia solani*, and *Trichoderma* sp. Antifungal activity was then determined by the diameter of zones within which fungal growth was inhibited.

RESULTS

BS061 controls powdery mildew under greenhouse conditions. We performed greenhouse experiments showing that the natural disease occurrence during the experiment was 78% of disease severity, indicating a heavy

Table 1. Control effect of *Bacillus* sp. BS061 against cucumber powdery mildew in greenhouse

	Disease incidence (%)	Control effect (%)
Culture filtrate[a]	29.3 ± 5.8	62.4
Competitor A[b]	34.4 ± 6.9	55.9
Fenarimol 4,000×	5.7 ± 2.8	92.7
Control	78 ± 9.3	-

Data were obtained 7 days after the final treatment. (±) represent the standard deviation from five replications.
[a]Twenty-fold dilution of culture filtrate was sprayed onto whole plants.
[b]A commercial product for the biocontrol of gray mold and powdery mildew.

Table 2. Control effect of *Bacillus* sp. BS061 against powdery mildew of strawberry in greenhouse

Treatment	No. of fruits		Disease incidence (%)	Control effect (%)
	Total	Diseased		
Culture filtrate[a]	459	74	16.1	80.3
Competitor A[b]	376	138	36.7	55.1
Triflumizole 4,000×	431	56	13	84.1
Control	486	397	81.7	-

The number of infected fruits was calculated 7 days after the final treatment.
[a]Twenty-fold dilution of culture filtrate was sprayed onto whole plants.
[b]A commercial product for the biocontrol of gray mold and powdery mildew.

infection of powdery mildew on the cucumber plant. Treatment with the culture filtrate of *Bacillus* sp. BS061 produced a disease control value of 62.4%, which was more effective than a biofungicide, which had a disease control value of 55.9% (Table 1). The disease incidence of strawberry fruit during natural disease occurrence was compared between synthetic fungicide treatment, biofungicide treatment, and no treatment (Table 2). The disease control effect of *Bacillus* sp. BS061 on strawberry fruit was 80.3%, which was as efficient as the synthetic fungicide triflumizole (84.1%) in the control of powdery mildew disease.

BS061 controls gray mold in tomato seedlings. The control effects of *Bacillus* sp. BS061 against *B. cinerea* in tomato seedlings are summarized in Fig. 1A. Treatment with the culture filtrate remarkably decreased disease incidence, with 90% control efficacy after 7 days of incubation at 20°C. The cell concentrations of BS061 significantly influenced the disease incidence of gray mold in tomato seedlings (Fig. 1B); higher concentrations of the antagonist resulted in better biocontrol effects. When *Bacillus* sp. BS061 was 1×10^8 CFU/mL, the best control effect was obtained and the occurrence of gray mold was significantly reduced compared with treatments at other concentrations.

The culture filtrate of *Bacillus* sp. BS061 has antifungal activity. *Bacillus* sp. BS061 was tested for its ability to antagonize the growth of a wide variety of pathogenic plant fungi responsible for diseases of economic importance. The antifungal activities of the culture filtrate of strain BS061 against 12 pathogenic fungi are provided in Table 3. The culture filtrate exhibited potent antifungal activity against *M. grisea*, *F. oxysporum*, *F. fulva*, *A. mali*, *C. gloeosporiodes*, *A. panax*, *C. destructans*, and *R. solani*.

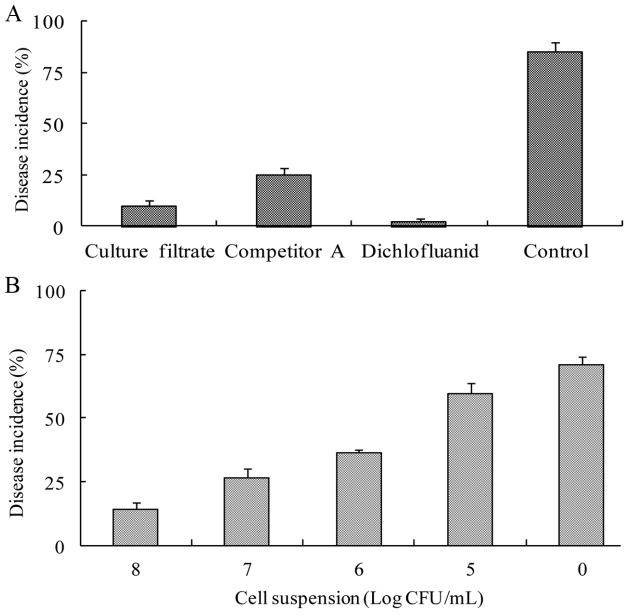

Fig. 1. Control effect of *Bacillus* sp. BS61 against gray mold on tomato seedlings. Twenty-fold dilutions of the culture filtrate (A) and the cell suspension (B) were sprayed onto whole plants. Disease incidence was measured 7 days after the final treatment with a *B. cinerea* spore suspension. Error bars represent the standard deviation from three replications.

Table 3. Antifungal activity of the culture filtrate from *Bacillus* sp. BS061

Fungal pathogen	Mycelium growth inhibition (mm)
Botrytis cinerea	15 ± 1.4
Magnaporthe grisea	21.5 ± 2.1
Fusarium oxysporum	15 ± 1.9
Fulvia fulva	13.5 ± 1.1
Collectotrichum gloeosporiodes	27 ± 2.3
Alternaria mali	17 ± 1.5
Alternaria panax	28 ± 1.5
Phytophthora capsici	0
Pythium sp.	0
Cylindrocarpon destructans	11 ± 1.3
Rhizoctonia solani	10 ± 1.5
Trichoderma sp.	0

DISCUSSION

Bacillus species have been reported to be effective for the biological control of multiple plant diseases due to their production of several broad-spectrum antibiotics and their longer shelf lives from their ability to form endospores [20, 22]. Antibiotic production plays a major role in disease suppression from these strains [12]. Our screening program for microorganisms with the potential to be used as microbial fungicides led us to a *Bacillus* species strain, BS061, which potently inhibited the mycelial growth of *Botrytis cinerea*. Treatment with BS061 culture filtrate was effective for controlling the development of gray mold caused by *B. cinerea*. In addition to its activity against gray mold, BS061 significantly reduced the disease incidence of powdery mildew in cucumber and strawberry plants under glasshouse conditions. The control effect of a twenty-fold diluted culture filtrate of *Bacillus* sp. BS061 against powdery mildew and gray mold was just as efficient as a synthetic fungicide. We are currently trying to purify antifungal compounds against gray mold and powdery mildew. Antifungal substances of the *Bacillus* sp. BS061 will be reported elsewhere.

Our results suggest that *Bacillus* sp. BS061 is a good biocontrol candidate. In conclusion, the application of *Bacillus* sp. BS061 may allow growers an opportunity to limit the use of chemical fungicides and permit biological measures for the control of powdery mildew and gray mold. Future research will be aimed at developing technology for the application of biocontrol agents to large-scale operations, and at investigating the mechanisms of strain BS061 for the control of gray mold and powdery mildew in plants.

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