Plant Pathol. J. 21(1) : 59-63 (2005)

The Plant Pathology Journal

Bacillus subtilis S1-0210 as a Biocontrol Agent against *Botrytis cinerea* in Strawberries

Nguyen Thi Thu Hang, Soon-Ok Oh, Gyoung Hee Kim, Jae-Seoun Hur[1] and Young Jin Koh*

Department of Plant Medicine and [1]Department of Environmental Education, Sunchon National University, Sunchon 540-742, Korea

(Received on October 1, 2004; Accepted on December 16, 2004)

***Bacillus subtilis* S1-0210 was selected as a biological agent against *Botrytis cinerea* in strawberry. The isolate inhibited mycelial growth of *B. cinerea in vitro* tests. A wettable powder formulation of *B. subtilis* S1-0210 significantly reduced infection rates with lower than 5%, compared with higher than 70% of infection rates in untreated control. The formulation showed 85 to 89% control efficacies of gray mold incidences on fruits of strawberry in pots. Pre-treatment of the agent was more effective in controlling gray mold on fruits and leaves than post-treatment at the early stage of disease development. The formulation also showed 70% control efficacy of gray mold incidence on fruits of strawberry in a field trial. The results indicate that *B. subtilis* S1-0210 in the wettable powder formulation may be a potential biocontrol agent to control gray mold on strawberry.**

Keywords **:** *Bacillus subtilis,* biocontrol, *Botrytis cinerea*, gray mold, strawberry

Strawberry gray mold, caused by *Botrytis cinerea,* is one of the most serious diseases that affects fruits, leaves, petioles, stems, and flowers in cold and wet weathers (Agrios, 1997). In addition, conidial spores contaminated during harvest can cause serious storage rot, especially when fruits are wet (Braun and Sutton, 1987). Gray mold disease on strawberry has been mainly controlled by application of chemical fungicides.

However, the emergence of resistant isolates to fungicides and the increasing consumer demand without fungicide residues have emphasized the need for development of alternative disease control strategies including biological control (Cook et al., 1996). Biological control of *B. cinerea* in strawberry was reported as one of the alternatives (Peng and Sutton, 1991; Sutton and Peng, 1994).

This evaluated *Bacillus subtilis* S1-0210 as a biocontrol agent against *B. cinerea* in strawberry. To evaluate its biocontrol activity *in vitro* on strawberry fruits during storage, *B. subtilis* S1-0210, formulated into a wettable powder, was used by the researchers on fruits and leaves in pots and fruits in a field trial.

*Corresponding author
Phone) +82-61-750-3865, FAX) +82-61-750-3208
E-mail) youngjin@sunchon.ac.kr

Materials and Methods

Isolation of antagonistic microorganisms. Strawberry leaves were collected from several strawberry farms around Sunchon area during winter growing season of 2002. The leaves were shaken with 50 ml of sterilized distilled water in a flask (250 ml) for 10 min, and heat-treated at 80°C for 20 min. Bacterial colonies were isolated 72 hrs after incubation of 100 ul of the suspension on nutrient agar (NA) plates at 28°C. Eighteen isolates were screened against 15 fungal pathogens. Mycelial disks of freshly grown fungal pathogens were incubated with bacterial isolates on the same plates. Inhibition zones of mycelial growths of the fungal pathogens were rated 7 days after incubation at 28°C.

Identification of the selected bacterial isolate. S1-0210 which showed promising results as a biological control agent, was selected for identification. Taxonomic schemes and criteria to determine the isolate were followed by Bacteriology Committee of the American Phytopathogenical Society (Schaad, 1988). The 16S rDNA sequence of S1-0210 was analyzed and aligned with the reference sequence by using NCBI BLAST searching tool.

Formulation of a wettable powder. S1-0210 isolate was incubated in 5 L of nutrient broth (NB, Difco, USA) containing 0.5% peptone (w/v) and 1% glucose (w/v) at 25°C for 5 days. The cells were centrifuged at 10,000 rpm for 15 min and resuspended into 125 ml NB. A wettable powder was formulated with cell suspension and inert ingredients such as bean flour, rice flour, glucose, $FeSO_4 \cdot 7H_2O$, and $MnCl_2 \cdot 4H_2O$. All of these compounds were thoroughly mixed and dried at 55°C for 36 hrs, and then pulverized. The formulated product contained more than 10^8 CFU/g of antagonistic bacteria. In addition, a blank formulation without the bacterial cells was also prepared.

All formulations were stored at 4°C until use.

***In vitro* bioassay of the wettable powder.** The wettable powder was treated on half of a glucose agar (glucose 10 g, beef extract 10 g, peptone 5 g, NaCl 5 g, agar 20 g, distilled water 1,000 ml) plate. Freshly grown mycelial disc (8 mm diameter) of *B. cinerea* was placed on the other half of the plate. The inhibition zone of mycelial growth of *B. cinerea* was rated 7 days after incubation at 28°C. This was then compared with the mycelial growth of *B. cinerea* treated with control agent. The bacterial isolates showing antagonistic activity against *B. cinerea* on the plates were reisolated and reconfirmed with 16S rDNA sequence analysis.

Evaluation of antagonistic activity on strawberry fruits during postharvest. Conidial suspension (10^5 conidia/ml) of *B. cinerea* was prepared and inoculated on the healthy strawberry fruits. Pre- or post-application of the wettable powder was performed 48 hrs before or after artificial inoculation with the conidial suspension until runoff. Conidial inoculation without the formulation was also treated as a control. Inoculated fruits were incubated for 5 days at 20°C. The number of fruits showing infection symptom was recorded to determine control efficacy.

Evaluation of antagonistic activity of the wettable powder in pots. The seedling plants were transplanted in October 2003 and cultivated under glasshouse condition for 2 months. Three plants were grown in a pot and each treatment contained 5 pots with 3 replicates. The wettable powder was applied 2 times with a 1-week interval at the flowering stage, when most primary berries were at green maturing stage. A suspension of approximately 10^6 CFU/ml and the diethofencarb-carbendazim WP fungicide (500 mg/L) were spayed on leaves and flowers until runoff. Conidial suspension (10^5 conidia/ml) of *B. cinerea* was sprayed 3 days before or after the formulation until runoff and maintained under 20°C and over 90% RH. Number of diseased strawberry fruits and leaves was determined 3 times with a 1-week interval 21 days after inoculation.

Evaluation of antagonistic activity of the wettable powder in field trial. Strawberry plants were grown under controlled condition that maintains a temperature range at 5 to 25°C and 80 to 90% RH. Neither fungicides nor pesticides were applied to the plants during the whole experimental period to control other diseases or insect pests. Seven plants were placed per meter ridge and approximately 20 plants were grown in a plot with 3 replicates. The wettable powder of approximately 10^6 CFU/ml and diethofencarb-carbendazim WP fungicide (500 mg/L) were sprayed with 0.05% (v/v) Tween-20 and 0.5% (v/v) Glycerin until runoff. The application was performed once a week from February 13 to March 8, 2004. Number of diseased strawberry fruits was determined 21 days after last application.

Results and Discussions

Isolation and identification of antagonistic agent. Eighteen endospore-forming isolates were selected on nutrient agar plate. Among the isolates, 11 isolates inhibited mycelial growth of *B. cinerea* by more than 60%. In particular, the isolate S1-0210 showed the highest inhibition rates (Fig. 1A). The isolate caused abnormal mycelial growth of *B. cinerea* (Fig. 1B). The isolate also inhibited mycelial growth of 15 plant pathogenic fungi (Table 1). The isolate was identified as *B. subtilis* based on biochemical, morphological, and cultural characteristics. The same isolate showed identical characteristics with those of the authentic strain (Table 2). The isolate was also confirmed with 16S rRNA sequences analysis using NCBI BLAST searching tool. The sequence showed more than 99% homology with the reference one.

***In vitro* bioassay.** The wettable powder exhibited a stable viability and antagonistic activity against *B. cinerea* on glucose agar (Fig. 2).

Evaluation of antagonistic activity on stored strawberry fruits. The wettable powder effectively controlled gray mold on strawberry fruits during postharvest (Fig. 3). The

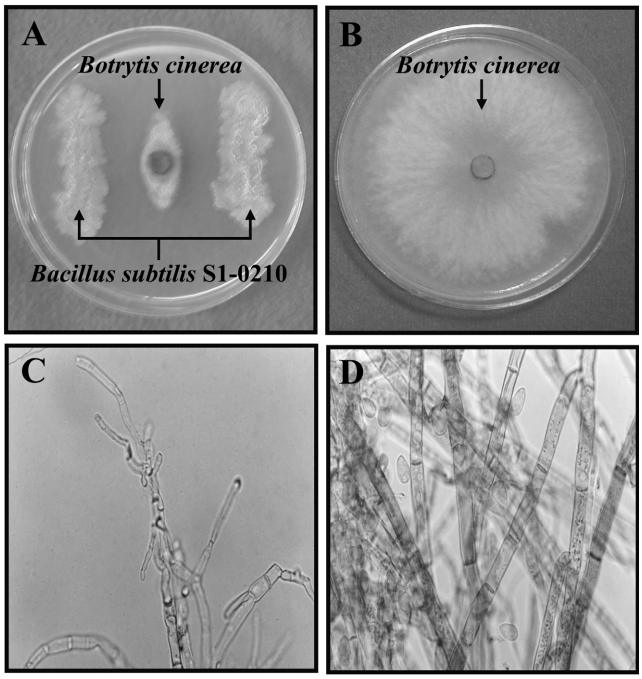

Fig. 1. *In vitro* inhibition assay of the selected bacterial isolate S1-0210 against *Botrytis cinerea*. **A**, Inhibition of mycelial colony of *B. cinerea* by the antagonistic bacterial isolate 7 days after incubation on glucose agar, **B**, Normal mycelial colony of *B. cinerea*, **C**, Abnormal mycelia of *B. cinerea* inhibited by the antagonistic bacterial isolate, **D**, Normal mycelia of *B. cinerea.*

Table 1. Antifungal activity of *Bacillus subtilis* S1-0210 against various plant pathogenic fungi on nutrient agar plates

Pathogen	Radius of mycelial colony (mm)[a]		Inhibition (%)
	Untreated control	S1-0210	
Bipolars coicis	16.2 ± 1.4	9.6 ± 1.0	41
Botryosphaeria dothidea	28.7 ± 1.7	11.2 ± 1.6	61
Botrytis cinerea (kiwifruit)	32.4 ± 1.0	11.2 ± 0.7	66
B. cinerea (strawberry)	30.3 ± 0.7	6.0 ± 0.9	80
Cercospora kikuchii	15.3 ± 0.8	7.3 ± 0.4	52
Colletotrichum coccodes	15.4 ± 0.5	8.0 ± 1.2	48
Diaporthe actinidiae	18.8 ± 1.0	9.9 ± 0.8	47
D. medusae	18.5 ± 0.8	8.8 ± 1.0	53
Fusarium graminearum	25.9 ± 0.1	12.2 ± 1.3	53
Glomerella cingulata	21.9 ± 1.2	7.6 ± 0.6	65
Magnaporthe grisea	13.8 ± 0.7	7.1 ± 1.0	49
Pestalotiopsis longiseta	21.0 ± 2.7	9.8 ± 1.6	54
Phomopsis soje	15.6 ± 2.5	7.7 ± 0.5	51
Pythium sp.	42.0 ± 1.7	20.4 ± 2.0	51
Rhizoctonia solani	32.8 ± 0.8	17.1 ± 2.1	48

[a] Measured 7 days after incubation.

Table 2. Biochemical, morphological, and cultural characteristics of *Bacillus* isolate S1-0210 compared with *Bacillus subtilis*[a]

Characteristics	*Bacillus subtilis*	Isolate S1-0210
Gram reaction	+	+
Endospore	+	+
Motility	+	+
Oval spore	+	+
Swelling of bacillary body	–	NT[b]
Flagella	–	–
Anaerobic growth in glucose broth	–	–
Spore position		
Terminal	–	–
Central	+	–
Sub-terminal	–	+
Growth at 15°C	+	+
25°C	+	+
35°C	+	+
45°C	+	+
Growth at pH 5.7	+	+
Growth in 7% NaCl	+	+
2% NaCl	+	+
5% NaCl	+	+
Utilization of citrate	+	+
Starch hydrolysis	+	+

[a] Schaad et al. (2001).
[b] NT: not tested.

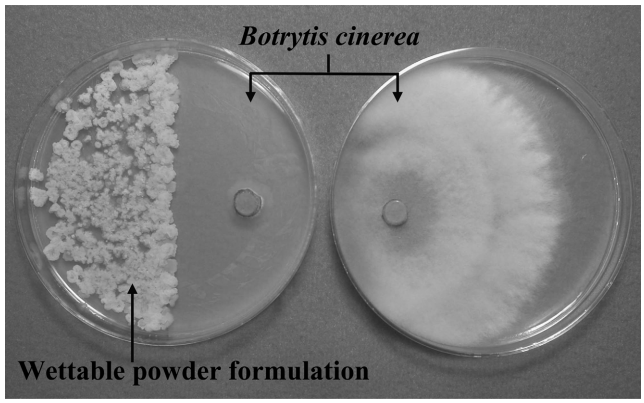

Fig. 2. Inhibition of mycelial growth of *Botrytis cinerea* by the wettable powder formulation of *Bacillus subtilis* S1-0210 after 7 days incubation on glucose agar.

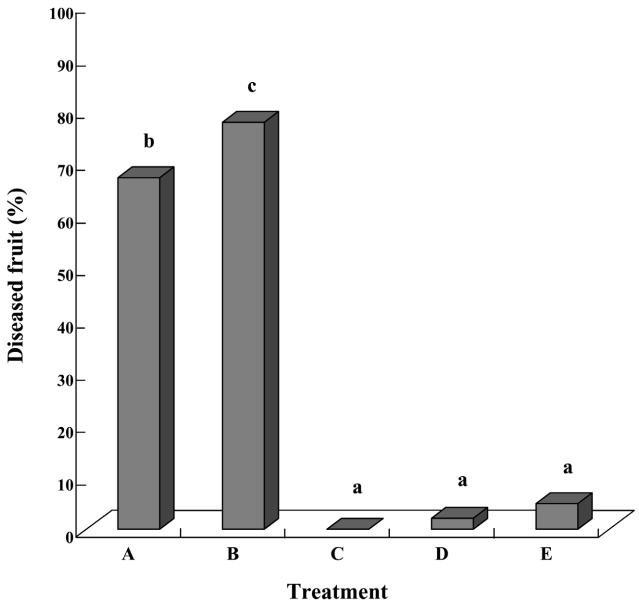

Fig. 3. Control efficacy of the formulated agent of *Bacillus subtilis* S1-0210 against the gray mold on strawberry fruits during storage. **A**, Treatment of the wettable powder formulation without S1-0210 isolate, **B**, Untreated control, **C**, Treatment of the wettable powder formulation without inoculation of *Botrytis cinerea*, **D**, Treatment of the wettable powder formulation 2 days before inoculation of *B. cinerea*, and **E**, Treatment of the wettable powder formulation 2 days after inoculation of *B. cinerea*. Conidial suspension (10^5 conidia/ml) of *B. cinerea* was sprayed on the healthy strawberry fruits until runoff. Means followed by the same letter are not significantly different by Duncans multiple range tests at $P = 0.05$.

formulation significantly reduced infection rates with lower than 5% infection occurring, compared with higher than 70% of infection rates exhibited by the fruits in untreated control.

Evaluation of antagonistic activity in pots. The wettable powder also effectively controlled gray mold on leaves and

Table 3. Effect of the wettable powder formulation with *Bacillus subtilis* S1-0210 against strawberry gray mold in pots

Treatment[a]	Disease incidence (%)[b]					
	21 DAI[c]		28 DAI		35 DAI	
	Fruit	Leaf	Fruit	Leaf	Fruit	Leaf
Control	5.3a	0.0a	6.0a	0.0a	8.0a	0.0a
Pathogen alone	92.6b	70.6b	89.7c	89.9b	93.1b	93.3c
WP + pathogen	2.9a	0.0a	17.1b	0.0a	11.4a	0.0a
Pathogen + WP	4.0a	0.0a	22.2a	2.0a	12.1a	3.0b
Fungicide + pathogen	7.4a	0.0a	6.7a	0.0a	6.7a	0.0a
Pathogen + fungicide	4.0a	0.0a	3.6a	0.0a	7.1a	0.0a

[a] Control: Sterilized water was sprayed, Pathogen alone: *Botrytis cinerea* was inoculated. WP + pathogen: Wettable powder formulation was applied 3 days before inoculation of *B. cinerea*, Pathogen + WP: Wettable powder formulation was applied 3 days after inoculation of *B. cinerea*, Fungicide + pathogen: Diethofencarb-carbendazim WP was applied 3 days before inoculation of *B. cinerea*, Pathogen + fungicide: Diethofencarb-carbendazim WP was applied 3 days after inoculation of *B. cinerea*.

[b] Number of diseased fruits or leaves. The data represent the means of three replicates.

[c] Days after inoculation.

Means in a column followed by the same letter are not significantly different by Duncan's multiple range tests at $P = 0.05$.

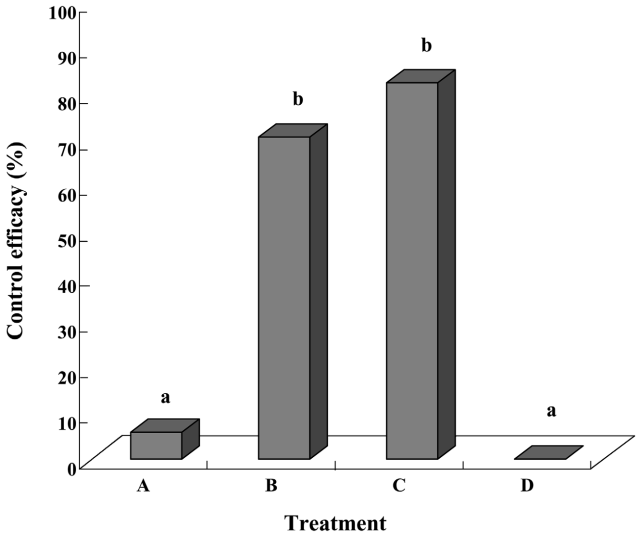

Fig. 4. Control efficacy of the wettable powder formulation of *Bacillus subtilis* S1-0210 against gray mold incidence on strawberry fruits during winter season 2004 (04/02/13 to 04/03/08) in the field trial. **A**, Treatment of the wettable powder formulation without S1-0210 isolate, **B**, Treatment of the wettable powder formulation, **C**, Treatment of the fungicide diethofencarb-carbendazim WP, **D**, Untreated control. The formulation was treated once a week during the period. Means followed by the same letter are not significantly different by Duncans multiple range tests at $P = 0.05$.

fruits of strawberry plants in pot experiment. At 35 days after inoculation, incidence of gray mold on fruits reached 93.1% in untreated control with inoculation of the pathogen only. However, infection rate of strawberry fruits was 12.1% only with the wettable powder, and 7.1% only with diethofencarb-carbendazim WP fungicide (Table 3). Disease incidence on leaves was also controlled in a similar manner with that of fruits. Pretreatment of the agent was more effective in controlling gray mold on fruits and leaves than post-treatment at the early stage of disease development.

Evaluation of antagonistic activity in the field trial. The wettable powder consistently exhibited strong antagonistic activity even in the field trial. The fruits of all plants in the untreated control were infected and showed typical symptom of gray mold. However, the wettable powder successfully controlled infection of *B. cinerea* on strawberry fruits with 70% control efficacy (Fig. 4). There was no significant difference in control efficacy between the fungicide and the wettable powder.

Efficacy of *B. subtilis*. Some potential antagonistic microorganisms selected through *in vitro* test often fail to effectively control pathogens in the greenhouse or in field trials (Weller, 1988). However, it was clearly demonstrated in this study that *B. subtilis* S1-0210 very effectively controlled gray mold on strawberry plants in the pot and the field trial. The effectiveness may be due to the followings: 1) the isolate originated from the same host plants grew in a similar environmental condition where the isolate was applied, 2) the isolate can form endospores which can ensure the stability and viability of the bacterial cells in harsh conditions, and 3) the isolate was likely specific to the pathogen.

Although *B. subtilis* S1-0210 inhibited mycelial growth of the fungal pathogen on agar plate, the antifungal activity was not mediated by the cell-to-cell contact. It is well known that a number of *Bacillus* strains can produce antibiotics. Examples include the lipopeptide family fengycin (Vanittanakom et al., 1986), zwittermycin A (an aminopolyol), kanosamine (3-amino-3-deoxy-D-glucose) (Silo-Suh et al., 1994), and peptides such as surfactin (Nakano et al., 1988) and subtilin (Klein et al., 1992). The isolate S1-0210 produces antibiotic substance which is diffused into the agar medium and causes abnormal shape of mycelia of *B. cinerea*.

Conclusion. Pre-application of the isolate before pathogen inoculation is more effective in controlling gray mold than post-application. Pre-colonization of antagonistic agent on host plants can be a critical factor in preventing the host from infection of fungal pathogen. In particular, this may be true for phyllosphere bacterium to compete fungal pathogens for common demands in a limited area. It is already proven with the commercialized agent of *Pseudomonas fluorescens* that prebacterization on the hosts successfully

controlls bacterial pathogens on aerial plant surfaces (Lindow et al., 1996).

The wettable powder formulation very effectively controlled gray mold. The formulation was easily suspended in water after slight agitation and was effectively applied through spray nozzles without clogging. The agent (approximately 10^6 CFU/ml) was comparable to the fungicide in controlling gray mold on strawberry plants. The formulated agent also kept its viability for more than 6 months in a dry condition at room temperature (data not shown).

In general, the formulated agent is easier to handle and preserve than living cell agent. Therefore, the wettable powder formulation with *B. subtilis* S1-0210 is a promising biological control agent against gray mold on strawberry plants. More research areas such as the interaction with indigenous leaf microorganisms, application methods, and visible detection of the isolate on the host need to be done for more effective and stable control of gray mold in the field.

References

Agrios, G. N. 1997. Plant Pathology. Academic Press, San Diego, CA, USA. 339-342 pp.

Braun, P. G. and Sutton, J. C. 1987. Inoculum sources of *Botrytis cinerea* in fruit rot of strawberries in Ontario. *Can. J. Plant Pathol.* 9:1-5.

Cook, R. J., Bruckart, W. L., Coulson, J. R., Goettel, M. S., Humber, R. A., Lumsden, R. D., Maddox, J. V., McManus, M. L., Moore, L., Meyer, S. F., Quimby, P. C. Jr., Stack, J. P. and Vaughn, J. L. 1996. Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. *Biol. Control* 7:333-351.

Klein, C., Kaletta, C., Schnell, N. and Entian, K. D. 1992. Analysis of the genes involved in biosynthesis of the antibiotic subtilin. *Appl. Environ. Microbiol.* 58:132-142.

Lindow, S. E., McCourty, G. and Elkins, R. 1996. Interactions of antibiotics with *Pseudomonas fluorescens* strain A506 in the control of fire blight and frost injury to pear. *Phytopatholgy* 86:841-848.

Nagano, M. M., Marahiel, M. A. and Zuber, P. 1988. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *J. Bacteriol.* 170: 5662-5668.

Peng, G. and Sutton, J. C. 1991. Evaluation of microorganisms for biocontrol of *Botrytis cinerea* in strawberry. *Can. J. Plant Pathol.* 13:247-257.

Schaad, N. W., Jones, J. B. and Chun, W. 2001. *Plant Pathogenic Bacteria.* APS Press, Minnesota, USA. p. 373.

Silo-Suh, L. A., Lethbridge, B. J., Raffel, S. J., He., H., Clardy, J. and Handelsman, J. 1994. Biological activities of two fungistatics produced by *Bacillus cereus* UW85. *Appl. Environ. Microbiol*. 60:2023-2030.

Sutton, J. C. and Peng, G. 1994. Biocontrol of *Botrytis cinerea* in strawberry leaves. *Phytopathology* 84:615-621.

Weller, D. M. 1988. Biological control of soil-borne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* 26:379-407.

Vanittanakom, N., Loeffler, W., Koch, U. and Jung, G. 1986. Fengycin – a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. *J. Antibiotics* 39:888-900.