Journal of Microbiology and Biotechnology
Vol. 5. No. 6, 341-345, 1995

Biological Control of Powdery Mildew by Antibiotic-producing Microorganisms Antagonistic to *Erysiphe graminis*

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Seventy four microorganisms, which have antagonistic activity against to *Fusarium culmorum*, were tested for their inhibitory effect on colony development of obligate biotroph *Erysiphe graminis* f. sp. *hordei* Marchal, the causal agent of powdery mildew on barley plants. Of these, 13 actinomycetes isolates were shown to reduce the colony development of mildew completely by application of their 10% cell-free culture filtrates on barley leaves. An Isolate, A252, was the most powerful antagonist and its antifungal activity was further assessed. The colony development of mildew was significantly reduced by application of the 1% cell-free culture filtrate of isolate A252. In comparison to the control, the protective and curative application of 10% cell-free culture filtrate from A252 showed 88.5% and 96.1% reduction of colony numbers respectively. By the protective application, 68.3% of the inhibition was observed after 9 days of treatment, thus showed prolonged inhibitory effect. *In vitro* test, complete inhibition of the mycelial growth of *Microdochium nivale* was achieved by the treatment of 1% A252 culture filtrate and 80.2% of inhibition was observed by the 0.1% treatment.

In general, the mechanisms of biological control of plant diseases are classified as competition, parasitism/predation, and antibiosis (2, 6). Antibiosis is defined as an interaction between organisms whereby a metabolic agent produced by one organism exerts a harmful effect on the other and play a major role in the management of plant pathogens (8). This antibiosis is established by specific or nonspecific metabolites, lytic agents, enzymes, volatile compounds, or other toxic substances (15). Antibiotics are generally considered to be organic compounds of low molecular weight produced by microbes (8). Many bacteria, actinomycetes and fungi have been shown to produce antibiotics (23) and cell-free culture filtrates or extracts of these filtrates have been also used to demonstrate the possible role for antibiosis in bio-control of the plant diseases (8). The searches for new biological fungicides have been intensively made for better control of bacterial and fungal diseases.

Powdery mildew, caused by the obligate biotroph *Erysiphe graminis* f. sp. *hordei* Marchal, is a major foliar disease of barley in temperate regions (17, 22). The principal methods of controlling barley mildew are host resistance and systemic fungicidal application (3). However, years of breeding may be required to develop an acceptable resistant cultivar, and blanket application of fungicides to control plant diseases, not only increase environmental pollution (19) but also lead to development of undesirable resistant fungal pathogens (7). The purpose of this study was to screen antibiotic-producing antagonists to *E. graminis* on barley plants and evaluate their protective and curative effect on development of the mildew colony.

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Key words: biological control, *Erysiphe graminis*, actinomycetes

MATERIALS AND METHODS

Antagonistic Microorganisms

To isolate antibiotic-producing microorganisms antagonistic to powdery mildew, cell-free cultrue filtrates of the previousely isolated antagonists (16) were tested for activity against the development of mildew colony on barley plants in a growth chamber. The antagonists were grown in a 2% tryptic soy broth (pH 6.5) for 2-3 days in shaking incubator (27°C, 120 rpm). Cell-free culture filtrate and cells was prepared by centrifugation (12000×g for 30 min). The supernatant was filter-sterilized (0.4 μm) and the cell pellets were washed with sterilized water, followed by centrifugation and resuspended in sterile distilled water.

Plants and Pathogen

A culture of *E. graminis* DC f. sp. *hordei* Marchal (race C17 amsel) as a pathogen for powdery mildew

was maintained onto the susceptible barley cultivar Golden Promise in a growth chamber. For the mildew propagation, 20 seeds of Golden Promise were planted in a 350-ml pot (9×9 cm). They were grown at 5000 lux (HQL-lamp) for 16 h, with a day temperature of 20±1°C and a relative humidity of 50%, and a night temperature of 16±1°C and a relative humidity of 80%. Seven day-old seedlings were heavily inoculated with the stock isolate and grown as described above. After six days of inoculation, the plants were shaken to remove excessive old mildew conidia so that those in the inoculum, used 24 h later, were of equal age.

Seven seeds of Golden Promise were planted in a 250-ml pot (7×7 cm) with in a row and grown at the condition as described above. These plants were used as test plants in this study.

Treatments of Cell-free Culture Filtrate

Seven days after sowing, cell-free culture filtrates were treated onto leaves prior to inoculate the mildew conidia. Among 7 plants in a pot, one plant in the middle of the row was treated with water as a control and the other plants were exposured to the cell-free culture filtrate of the test antagonists. All treatments were applied to the leaves until runoff.

Measurement of Disease Suppression Effect

After 2 h from the treatments with antagonists, the back side of treated leaves were challange-inoculated with 2.5 mg conidia of *E. graminis* in a inoculation chamber (90×40×40 cm) for 3 minutes, the plants were transferred to the growth chamber. After 5 days of inoculation, the number of colonies on the leaves were counted and the disease suppression effect (SE, %) was expressed as percentage reduction in the number of colonies, compared to the control as follows;.

$$SE\,\% = (1 - \frac{\text{number of colony on treated leave}}{\text{number of colony on untreated leave}}) \times 100$$

Protective and Curative Effect of the Cell-free Culture Filtrate

Protective effect of the cell-free culture filtrate (CF) applications on the development of mildew colony was estimated by challange inoculation with mildew conidia after 3 days of CF treatment. For estimation of the curative effect of the CF, the CF was treated 3 days after inoculation with mildew conidia.

Antifungal Spectrum of Antagonistic Microorganisms

To examine the antifungal spectrum of the isolate A252, 6 phytopathogenic fungi species were used as targets. The cell-free filterate from the A252 isolate was filtered using a 0.45 μm membrane filter and then mixed with molten potato dextrose agar (PDA). The mycelial disk (5 mm in diameter) of test fungi were placed on the center of the petri dish (9 cm). After 4-7 days incubation at 27°C, the diameter of mycelium was measured and the relative inhibitory activity (RI, %) was determined as follows;

$$RI(\%) = (1 - \frac{\text{diameter of the mycelium on PDA containing CF}}{\text{diameter of the mycelium on PDA}}) \times 100.$$

RESULTS

Previously reported seventy four antagonistic microorganisms (16) were tested for their suppressing activities for the infection of *Erysiphe graminis* on barley plants in a growth chamber. Treatments with cell-free culture filtrate (10% in water) on barley leaves before inoculation with conidia of *E. graminis* resulted in 13 to 100% disease suppression as shown in Table 1 and Fig. 1. Their suppression effects were varied according to the antagonists. Among them, 10% CF from the 13 isolates of actinomycetes showed complete disease suppression, whereas most of the bacterial isolates result in low suppressing effects.

One percent of culture filtrates from 13 isolates was also tested for screening the best antagonistic actinomycetes and the results are shown in Table 2. Although the effect was dependent on the isolates, CFs from A156 and A252 showed the most suppressing activity for the development of the mildew colony. Some isolates (A110, A246, A254 and A267) showed low activity of suppression.

With five isolates (A8, A156, A206, A228 and A252) from the above, their protective and curative effects

Table 1. Suppression activities by antagonistic microorganisms on colony development of *Erysiphe graminis*[a].

Suppression effect (%)	Actinomycetes	Bacteria
0 - 20	0[b]	3
20 - 40	5	5
40 - 60	7	6
60 - 80	10	4
80 -100	17	0

[a]The cell-free culture filtrates (10%) of antagonists were treated by spraying on the leaves of spring barley cultivar Golden Promise at first leaf stage. After 2 h of treatment, conidia of *Erysiphe grammininis* were inoculated and the number of colony were counted after 4-5 days. The relative disease suppression effect (SE, %) was determined as follows;

$$SE\,\% = (1 - \frac{\text{number of colony on treated leaf/cm}^2\text{ leaf}}{\text{number of colony on untreated leaf/cm}^2\text{ leaf}}) \times 100\,.$$

[b]Number denotes the number of isolates grouped by similar disease suppression activities.

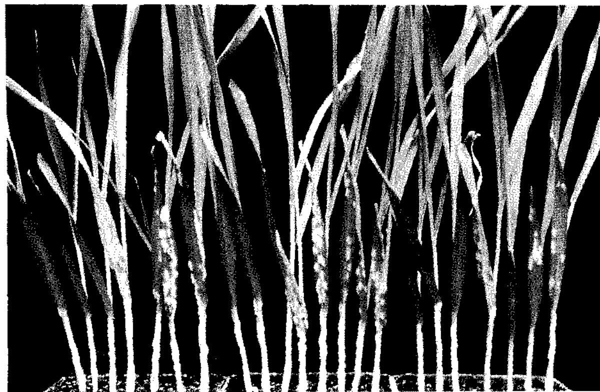

Fig. 1. The effect of cell-free culture filtrates from antagonistic microorganisms on colony development of powdery mildew on barley plants.
The cell-free culture filtrates (10%) of antagonists were treated by spraying on the leaves of spring barley cultivar Golden Promise at first leaf stage. After 2 h of treatment, conidia of *Erysiphe graminis* were inoculated. Photography were taken after 8 days inoculation.
C; control.

Table 2. Relative disease suppression effect of 13 actinomycetes on the colony development of powdery mildew on barley plants[a].

Antagonistic microorganisms	Density of colony (cm^{-2})	Disease suppression (%)
A8	12.1±1.5 bc[b]	52.2
A110	17.6±2.2 ef	30.5
A156	5.1±1.9 a	79.9
A197	13.3±2.7 c	47.5
A206	11.3±2.7 bc	54.4
A217	13.7±2.6 cd	45.9
A228	10.0±2.4 b	60.5
A246	16.8±3.0 ef	33.6
A252	4.3±1.4 a	83.1
A254	16.9±3.0 ef	33.3
A225	15.7±2.4 de	38.0
A260	13.3±2.4 c	47.5
A267	19.3±2.6 f	33.8
control	25.2±2.9 g	0

[a]Nine plants at one leaf stage were treated for 2 h with culture filtrate and inoculated with conidia of *Erysiphe graminis*. After incubation in 100% humidity condition for 4 h at 20°C follwed by 4 days in 60-70% relative hymidity at 20°C, the number of colonies per treatment was counted per corresponding leaf area. The relative disease suppression effect (SE, %) was determined as follows;

$$SE\ \% = (1 - \frac{\text{number of colony on treated leaf/cm}^2\text{ leaf}}{\text{number of colony on untreated leaf/cm}^2\text{ leaf}}) \times 100\ .$$

[b]Values followed by the same letter are not significantly different at p= 0.05.

were further examined. As shown in Table 3 and 4, A 252 showed 88.5% and 96.1% of the protective and curative effects, respectively, wherase those of A206 were less effective (34.6% and 61.0%). Based on this result A252 was selected as the most powerful antagonist against powdery mildew.

Table 3. Protective effect of the cell-free culture filtrate of 5 actinomycetes on the colony appearance of powdery mildew on barley plants[a].

Antagonistic microorganisms	Density of colony (cm^{-2})	Disease suppression (%)
A8	7.3±0.7 c[b]	70.0
A156	5.1±1.9 b	79.1
A206	15.9±0.7 d	34.6
A228	7.9±0.5 c	67.5
A252	2.8±1.0 a	88.5
control	24.3±2.2 e	0

[a]Ten percent cell-free culture filtrates of the antagonists were sprayed on 9 barely plants (cv. Golden Promise) at first leaf stage. Three days later, the plants were inoculated with conidia of *E. graminis*. After incubation in 100% humidity condition for 4 h at 20°C followed by 4 days at 60-70% relative hymidity at 20°C, the number of colonies per treatment on corresponding leaf area was counted. The relative disease suppression effect (SE, %) was determined as follows;

$$SE\ \% = (1 - \frac{\text{number of colony on treated leaf/cm}^2\text{ leaf}}{\text{number of colony 9on untreated leaf/cm}^2\text{ leaf}}) \times 100\ .$$

[b]Values followed by the same letter are not significantly different at p= 0.05.

Table 4. Curative effect of the cell-free culture filtrates of 5 actinomycetes on the colony appearance of powdery mildew on barley plants[a].

Antagonistic microorganisms	Density of colony (cm^{-2})	Disease suppression (%)
A8	2.9±1.2 b[b]	87.3
A156	3.2±1.1 b	86.0
A206	8.9±1.2 c	61.0
A228	2.4±0.9 b	89.5
A252	0.9±0.6 a	96.1
control	22.8±2.2 d	0

[a]The barley plants (cv. Golden Promise) were inoculated with conidia of *Erysiphe graminis* at first leaf stage (7 days after sowing). Three days later, 9 plants were treated with 10% cell-free culture filtrates of the antagonists. After five days, the number of colony per corresponding leaf area was counted. The relative disease suppression effect (SE, %) was determined as follow;

$$SE\ \% = (1 - \frac{\text{number of colony on treated leaf/cm}^2\text{ leaf}}{\text{number of colony on untreated leaf/cm}^2\text{ leaf}}) \times 100$$

[b]Values followed by the same letter are not significantly different at p= 0.05.

Furthermore, we tested the protective effect on mildew development during 9 days caused by the 10% CF and washed cells from A252. As shown in Table 5, the developments of mildew colonies from inoculated with conidia were inhibited to 84.3, 79.1 and 68.5% after 5, 7 and 9 days of treatments, respectively, by protective treatment of 10% CF. When the cell suspension was protectively treated before 9 days of mildew inoculation, mildew development was also inhibited to 60.4%.

The antifungal activity of A252 CF was further tested against the mycelial growth of six different phy-

Table 5. Protective effect of A252 on the colony appearance of powdery mildew on barley plants[a].

Inoculation time after treatment	Control	A252			
		Culture filtrate		Cell suspension	
2 h	25.3±2.9	0.0±0.0	100	0.0±0.0	100
1 day	24.1±4.1	0.0±0.0	100	0.0±0.0	100
3 days	24.3±2.2	2.8±1.0	88.5	3.1±1.3	87.3
5 days	21.0±4.1	3.3±1.4	84.5	2.7±1.5	87.5
7 days	18.1±3.3	3.8±1.2	79.1	4.7±1.9	74.2
9 days	16.5±3.2	5.2±1.1	68.5	6.5±1.3	60.4

[a]Ten percent cell-free culture filtrate of the isolate A252 and its cell suspension (OD660 nm=1.0) were sprayed on barely plants (cv. Golden Promise) at first leaf stage. Nine plants treated were then inoculated with conidia of *E. graminis* at different time. After incubation in 100% humidity condition for 4 h at 20°C follwed by 4 days in 60-70% relative humidity at 20°C, the number of colonies pertreatment on corresponding leaf area was counted. The relative disease suppression effect (SE, %) was determined as follows;

$$SE\ \% = \left(1 - \frac{\text{number of colony on treated leaf/cm}^2\text{ leaf}}{\text{number of colony on untreated leaf/cm}^2\text{ leaf}}\right) \times 100.$$

Table 6. Relative inhibition rate of the cell-free culture filtrate of A252 to mycelial growth of different plant pathogenic fungi[a].

Plant pathogenic fungi	Relative inhibition rate (Concentration of cultrue filtrate (%))			
	10	1.0	0.1	0.01
Fusarium culmorum	89.2[b]	77.4	48.1	17.2
Fusarium graminearum	77.8	69.4	33.2	8.2
Microdochium nivale	100.0	100.0	80.2	22.4
Phytophthora capsici	74.5	53.2	12.3	0.0
Pythium ultimum	97.6	59.4	16.8	0.0
Rhizoctonia solani	100.0	57.3	39.8	0.0

[a]A mycelial disk 5 mm in diameter of the actively growing test fungi were placed on potato dextrose agar containing cell-free culture filtrates with different concentration*.
[b]After 2-7 days incubation at 27°C, mycelial growth in millimeter was compared in the presence (a) or absense (c) of the culture filtrate. Inhibition rate (%) was caculated as follows;

$$\text{Inhibition rate(\%)} = \left(1 - \frac{a}{c}\right) \times 100.$$

topathogenic fungi on PDA. As shown in Table 6, PDA containing 0.01% CF of A252 was not shown inhibitory effect against *Phytophthora capsici, Rhizoctonia solani, Pythium ultimum,* while a slight inhibitory effect (22.4%) was observed in *Microdochium nivale.* However, at the 10% concentration of A252 CF, the growth of *P. capsici* was severely inhibited to 74.5%, whereas the complete inhibition were noticed in *M. nivale* and *R. solani.*

DISCUSSION

Blanket application of fungicides against target organisms to protect crops may lead to an undesirable development of resistant fungal pathogens at one end and elimination of normal saprophytes on the other, thereby providing the pathogens a clear advantage (14). As these chemicals are not often easily degradable, they increase environmental pollution (19). Increasing awareness of biological control of organisms and the plan to reduce pollution problems provide the greatest stimulus to explore the possibility of exploiting the capacity of the organisms to control fungal diseases biologically (5).

Biocontrol of foliar fungal pathogens received less attention than those of soil-borne pathogens (1, 14). However, many fungi, bacteria and actinomycetes were isolated from phylloplane or other habitat of foliar pathogens and applied as agents for biocontrol of foliar pathogens (6, 10, 14). Introduction of antagonists or their metabolites on the phylloplane is one approach to the control of filiar disease.

It has been well known that many bacteria, actinomycetes and fungi produce a variety of antibiotics which, in many cases, have been the postulated mechanism of an action against phytopathogens (4, 6, 9, 12, 13, 18, 20, 21). In our experiments, the culture filtrates tested from all antagonists showed inhibitory effect on the development of mildew colony in the range from 13 to 100%. This inhibition is believed to be due to the presence of inhibitory metabolites secreted from the microorganisms detrimental to mildew development. At present, about 8,000 antibiotics are reported, most of which are known to be produced by actinomycetes, especially by the *Streptomyces* sp. (15). The most actinomycetes tested in our experiment showed high suppression effect on the colony development of the mildew. Ten percent culture filtrates of 13 actinomycetes completely inhibited the development of the mildew. One percent culture filtrates from these isolates, however, resulted in different suppression effects. The reason might be due to the differences in amounts of antibiotics produced from each isolate. Most of bacterial isolates, in comparison, showed low antagonistic effect. Many antibiotics are used as agrochemicals and they are protective as well as curative for phytopathogens, being transferred in the plant body systematically (11). In our experiment, the effect of culture filtrate and cell suspension of A 252 were mainted during 9 days as protective activity and 3 days as curative activity, which were determined by measuring the suppression activity for the development of mildew conidia. Meanwhile, the culture filtrate of isolate A252, *in vitro* application showed a broad range and high level of antifungal activity. These results may be due to antibiosis by Cook and Baker (6), defined as "the inhibition or destruction of

one organism by a metabolic product of another."

The maintenance of inhibitory effect on the development of mildew colony by the protective and curative application suggests that the isolate A252 could be used as an effective candidate for mildew control practices. For more effective control program, further additional studies will be carried out such as properties of purified antibiotic, its antagonistic mode, and host reaction.

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(Received September 25, 1995)