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Isolation of Antibiotic-producing Bacteria Antagonistic to *Fusarium oxysporum* from Sesame-growing Soils and Evaluation of Their Antifungal Activity

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For isolation of antibiotic-producing bacteria antagonistic to *Fusarium oxysporum*, a total of 327 microorganisms were screened from sesame-growing soils collected at various locations in Korea by the modified Herr's triple-agar-layer technique. Among the 36 bacterial isolates further screened by the dual culture test on tryptic soy agar, 10 were tested to show their antagonistic activity against 14 plant pathogenic fungi. Bacterial culture filtrates were shown either to inhibit some phytopathogenic fungal growth or to suppress *F. oxysporum* infection of sesame plants maintained in the green house. An isolate, B23, with the most prominent antagonistic activity was identified as *Bacillus subtilis*.

In general, the mechanisms of biological control of plant diseases are classified as competition, parasitism/predation, and antibiosis (2, 5). Of these mechanisms, antibiosis plays a major role in the management of plant pathogens (2, 7, 26). Antibiotic chemicals originated from microorganisms may selectively inhibit soil microflora and have highly selective activity against some plant pathogens. Based on this, innovative natural agrochemicals have been intensively searched for to improve control of plant pathogens (3, 12, 25, 26).

Soilborne plant diseases caused by *Fusarium* sp., *Rhizoctonia* sp., *Phytophthora* sp., and *Pythium* sp. are considered to be the most destructive to growth of many of plant species. However, no effective control method has been developed. Fusarium wilts are soilborne plant diseases that cause serious problems in several plants. Because of the difficulties encountered in eradicating Fusarium wilt pathogens by conventional methods, biological controlling agents have been sought as an attractive and useful alternative (1, 15, 16, 18). Fusarium wilt of sesame plants caused by *Fusarium oxysporum* f. sp. *sesami* is a notorious soile-borne disease causing major losses in sesame crop yield (24). There are not only few effective chemical control methods, but fewer biocontrol studies have been done (4, 24).

The objectives of this study were to screen antibiotic-producing microorganisms against *F. oxysporum* f. sp. *vasinfectum* from the soil using adequate screening procedures (12) and to evaluate their antibiotic activity *in vitro* and *in vivo*.

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Key words: *Fusarium oxysporum*, antagonistic bacteria, biological control, *Bacillus subtilis*

MATERIALS AND METHODS

Screening for Antibiotic-producing Bacteria

Microorganisms antagonistic to *Fusarium oxysporum* were isolated according to the modified Herr's method (10). Soil samples collected from the rhizosphere 10 to 15 cm beneath the soil surface were obtained from several sesame-cultivating fields at different locations in Korea. Samples were stored at 4°C in plastic bags until their use.

Samples of 5 g of soil diluted with 50 ml of sterile water were thoroughly dispersed by shaking at 150 rpm for 30 min and then diluted 10^3 to 10^5 fold. One ml of the diluted sample was mixed with 9 ml of molten 2% tryptic soy agar (TSA, 2% tryptic soy broth, 1.5% agar, pH 6.5). After solidification, 5 ml of sterile water agar (1.2%) were added and incubated for 48 h at 27°C. 5 ml of potato dextrose agar containing conidia of *F. oxysporum* (10^4/ml) were added as the third agar layer. After another 48 h of incubation at 27°C, bacterial colonies with a surrounding inhibition zone were selected as primary candidates. For further screening, the isolated colonies were double streaked 4 cm wide on a TSA plate and incubated for 48 h at 27°C. A mycelial disk (5 mm in diameter) from the marginal region of *F. oxysporum* solid cul-

ture was placed in the middle of the double streaks and incubated for 5 days at 27°C. Bacterial isolates with relatively strong antagonistic activity to *F. oxysporum* were selected and stored at 4°C on soil extraction agar at pH 6.5 (13). For the preparation of soil extract agar, 300 g of a field soil was autoclaved in 500 ml distilled water, filtered, diluted 1 : 1 (v/v) with distilled water, and 1.5% Bacto agar (Difco) was added.

Antimicrobial Activities of the Bacterial Isolates

The extent of antagonistic activities against *F. oxysporum* of selected bacterial isolates was measured on both TSA and PDA media. The bacterial isolates grown in TSB (2% tryptic soy broth, pH 6.5) for 24 h at 27°C were streaked in 4 cm-long inoculates on one side of the plate. After two days, a mycelial disk 5 mm in diameter of *F. oxysporum* was placed 3.5 cm in distance from the streak. After 6 days incubation, the mycelial growth in centimeters both toward antagonistic bacterial streaks (a) and in the opposite direction (b) were measured. The fungal growth inhibition rate (IR, %) was determined as follows;

$$IR(\%) = 100 - (\frac{a}{b} \times 100)$$

To evaluate the antibacterial activity, test bacterium was streaked 10 mm from the isolate in a rectangular fashion, and inhibition zones were measured after two days.

Antifungal Activity of Culture Filtrates from the Bacterial Isolates

The bacterial isolates were grown in TSB for 3 days at 27°C and their cell-free filtrates were collected by centrifugation (7,000 g, 15 min) followed by aseptic 0.4 μm-filtration. The filtrates were stored at -20°C until their use. PDA containing 10% (v/v) culture filtrates was used to evaluate growth inhibition of test fungi. A mycelial disk (5mm in diameter) was placed in the center of the prepared medium and its growth was measured 1 to 21 days after incubation at 10 or 27°C.

A Greenhouse Test of Culture Filtrates

The culture filtrates were tested for their suppressive activity on the *F. oxysporum* infection of sesame plants maintained in the green house. Sesami cultivar Danbaek were grown in steam-sterilized soil mix (field soil : sand : loam soil, 1 : 1 : 1, v/v/v) in plastic pot. The pots containing the growing sesame were

Table 1. A list of antagonistic microorganisms isolated from sesame-growing soils.

Group	Anatagonistic bacteria	Colony morphology and color
I	B 1, B 2, B 3, B 4, B 5, B 9, B20, B22, B28, B39, B42	smooth slime, grayish white
II	B 6, B23, B24	rough, silvergray
III	B 7, B27, B36, B37	smooth fluorescent, pale yellow
IV	B10, B19, B26, B44	rough slime, purplish white
V	B13, B14, B16, B17, B40, B46, B48	rough slime, grayish white
VI	B21, B30, B31, B32, B33, B34, B43	smooth slime, purplish white

Table 2. Inhibition rates of mycelial growth of *Fusarium oxysporum* on the two media by various antagonistic bacteria isolated from sesame-growing soils[a].

Antagonistic bacteria	Inhibition rate (%) on the media	
	Tryptic soy agar	Potato dextrose agar
B 1	42.9[b]	40.0
B 2	41.2	38.6
B 3	38.5	44.0
B 4	39.3	38.8
B 5	35.8	36.4
B 6	55.7	52.0
B 7	35.8	36.0
B 9	39.3	38.8
B10	46.5	47.8
B13	34.9	36.0
B14	43.2	44.0
B16	39.9	34.8
B17	46.5	44.0
B19	46.5	44.0
B20	39.3	38.8
B21	34.5	36.4
B22	41.2	38.6
B23	53.6	52.0
B24	53.6	52.0
B26	43.0	44.0
B27	36.2	36.4
B28	39.3	38.8
B30	33.3	46.2
B31	45.8	46.4
B32	45.8	46.4
B33	42.9	37.6
B34	44.8	46.2
B36	26.7	30.4
B37	34.5	36.4
B39	39.3	40.0
B40	28.6	30.4
B42	35.8	36.4
B43	39.3	38.8
B44	34.5	36.4
B46	41.0	49.6
B48	38.5	38.8

[a]The antagonistic bacteria were streaked (40 mm) on one side of plates of the test media, and 48 h after incubation at 27°C mycelial disk (5 mm in diameter) of *Fusarium oxysporum* was placed at a 35 mm distant from the antagonistic bacteria. They were incubated at 27°C.

[b]After 6 days incubation at 27°C, the mycelial growth of *F. oxysporum* in distance (mm) of toward antagonistic bacteria (a), and distance in the opposite direction from antagonistic bacteria (b) were measured. The fungal growth inhibition rate (IR) was determined as follows;

$$IR(\%) = 100 - (\frac{a}{b} \times 100).$$

drenched with 50 ml of the culture filtrates containing spores of *F. oxysporum* (10^4/ml). After 14 days, disease incidence was assessed by counting sesame plants in a healthy condition and those with lesions.

Identification of Bacterium

Selected antagonistic bacterial strain was identified according to the methods described in Bergey's Manual of Systematic Bacteriology (14), Laboratory Manual of General Bacterology (9) and Analytical Profile Index System (API 50 CHB, bioMerieux, France).

RESULTS

Screening for Antibiotic-producing Microorganisms

A total of 327 microorganisms were screened from the rhizosphere or nonrhizosphere of various locations in Korea by the modified Herr's triple-agar-layer technique. Their antagonism against *Fusarium*

Table 3. Inhibition of mycelial growth of various plant pathogenic fungi on the tryptic soy agar by antagonistic bacteria isolated from soils of various locations in Korea[a].

Plant pathogenic fungus	Inhibition rate (%)[b]									
	B3	B6	B14	B16	B23	B26	B27	B30	B36	B46
Fusarium oxysporum f. sp. *sesami*	38.5	55.7	43.5	39.9	53.6	43.0	36.2	33.3	26.7	41.0
Fusarium oxysporum f. sp. *cucumerinum*	44.8	63.4	46.7	43.4	60.0	50.0	40.0	30.0	40.0	46.7
Fusarium moliniform	44.0	54.2	42.9	42.9	57.2	48.0	30.8	45.9	29.2	45.9
Fusarium roseum	44.6	63.4	46.7	44.6	56.7	55.2	44.6	55.2	10.8	48.2
Fusarium solani	39.7	52.6	32.3	39.7	50.2	40.5	34.4	42.9	32.3	44.6
Pythium ultimum	38.3	64.8	30.6	44.2	64.8	55.2	30.6	44.2	30.8	44.2
Pythium aphanidermatum	8.4	20.6	17.7	3.0	23.8	20.6	6.9	13.8	15.3	8.9
Rhizoctonia cerealis	42.4	92.4	57.7	46.2	92.8	73.1	30.0	41.7	88.2	42.9
Rhizoctonia cerealis	42.4	92.9	57.7	50.0	96.5	61.6	46.7	27.8	89.4	42.9
Rhizoctonia solani	43.4	99.9	59.4	39.3	99.9	71.5	34.4	27.6	63.4	43.4
Rhizoctonia solani	51.5	73.6	44.2	50.0	64.8	64.8	35.3	45.5	67.7	55.9
Phytophthora capsici	56.7	85.8	65.7	57.7	82.2	61.6	19.1	31.9	65.4	56.6
Rhizoctonia solani	18.8	53.2	31.3	21.9	58.9	38.3	13.0	41.2	46.9	32.3
Typhula incarnata	51.6	61.5	54.8	53.3	65.5	55.2	56.7	55.2	48.8	55.2

[a]The antagonistic bacteria were streaked (40 mm) on one side of plates of the test media, and 48 h after inocubation at 27°C a mycelial disk (5 mm in diameter) of the actively growing culture plate of the test fungi was placed 35 mm from the antagonistic bacteria. They were incubated at 10 or 27°C, depending on the species.

[b]After 2-21 days of incubation, mycelial growth distance in mm of the test fungi toward the antagonistic bacteria (a), and distance in the opposite direction from antagonistic bacteria (b) were measured. The fungal growth in hibition rate (IR) was determined as follows;

$$IR(\%) = 100 - (\frac{a}{b} \times 100).$$

Table 4. Antifungal activity of cell-free culture filtrates of antagonistic bacteria isolated from sesame-growing soils[a].

Plant pathogenic fungus	Inhibition rate (%)[b]									
	B3	B6	B14	B16	B23	B26	B27	B30	B36	B46
Fusarium oxysporum f. sp. *sesami*	2.0	66.7	6.7	6.7	66.7	8.9	0.0	4.5	0.0	12.0
Fusarium oxysporum f. sp. *cucumerinum*	0.0	62.3	11.2	4.5	62.3	6.7	0.0	6.7	0.0	14.0
Fusarium moliniform	4.0	68.9	4.0	4.0	68.9	4.0	0.0	4.5	0.0	8.0
Fusarium roseum	0.0	87.2	7.2	0.0	85.8	0.0	0.0	0.0	0.0	18.0
Fusarium solani	0.0	62.3	4.5	4.5	62.3	0.0	0.0	4.5	10.4	5.5
Pythium ultimum	21.5	73.9	38.5	20.0	73.9	0.0	0.0	20.7	0.0	21.5
Pythium aphanidermatum	0.0	10.4	5.8	0.0	13.8	17.0	6.9	13.9	3.5	8.5
Rhizoctonia cerealis	0.0	60.0	3.9	0.0	73.2	13.9	4.7	30.8	6.2	6.9
Rhizoctonia cerealis	0.0	69.3	3.9	4.5	69.3	20.7	7.7	47.2	6.2	4.0
Rhizoctonia solani	0.0	58.5	6.7	0.0	64.6	47.3	17.0	10.0	6.7	0.0
Rhizoctonia solani	0.0	56.7	0.0	0.0	56.7	60.0	23.4	20.7	6.7	15.0
Phytophthora capsici	0.0	26.0	6.6	0.0	24.4	36.7	2.2	20.7	6.7	7.5
Rhizoctonia solani	5.9	53.4	8.0	4.8	68.6	18.3	6.7	0.0	0.0	8.6
Typhula incarnata	0.0	80.0	0.0	0.0	82.4	0.0	0.0	0.0	0.0	0.0

[a]A mycelial disk (5 mm in diameter) of the actively growing culture plate of the test fungi was placed on a potato dextrose agar plate (15 ml/plate) which contained 10 ml of cell free-culture filtrates of antagonistic bacteria isolates mixed with 90 ml molten potato dextrose agar.

[b]After 2 or 21 days incubation at 10 or 27°C, depending on the species, the diameter of the tested fungal mycelium (a) was measured, and the inhibition rate (%) was calculated from mycelial growth on the potato dextrose agar (control) as follow;

$$\text{Inhibitory rate } (\%) = 100 - (\frac{a}{\text{Control}} \times 100).$$

Table 5. Antibacterial activity of antagonistic bacteria on tryptic soy agar[a].

Target microorganisms	Inhibition zone length (mm)[b]									
	B3	B6	B14	B16	B23	B26	B27	B30	B36	B46
Bacillus subtilis	0	0	0	0	0	2	0	0	0	2
Corynebacterium rathayi	3	0	4	4	0	7	0	5	0	5
Erwinia carotovora	0	0	0	0	0	0	0	0	0	0
Eschericha coli	2	0	2	2	0	2	0	1	0	2
Pseudomonas fuloresens	0	0	0	0	0	0	0	0	0	0
Xanthomonas campestris	2	0	3	3	0	3	0	1	0	3
Saccharomyces cerevisiae	0	4	0	0	4	3	0	0	18	0

[a]Antibacterial activity was tested by streaking inoculates of target microoganisms rectangulary 10 mm from the antagonistic bacteria while the antagonistic bacteria isolates were inoculated on the side of the plate. They were incubated for 4 h at 27°C.
[b]Inhibition zones were measured 2 days after inoculation of target microorganisms.

oxysporum was assessed by the dual culture method, and, as a consequence, 36 bacteria and 48 actinomycetes were selected (data not shown). The bacterial isolates only were chosen for further examination. The selected antagonistic bacterial isolates were catagorized into 6 groups according to the characteristics of morphology and color on TSA (Table 1). Their antifungal activities depending on medium formulas such as TSA and PDA were also tested. Most isolates were not affected as shown in Table 2, but isolates B16 and B33 were more inhibitory on TSA. In contrast, PDA was more favorable for isolates B 3 and B46.

Antimicrobial Spectrum of the Bacterial Isolates

Among 36 bacterial isolates, ten were examined for their antagonistism against 13 pathogenic fungal species. The results are shown in Table 3. In particular, the isolates B6 and B23 demonstrated stronger inhibition of mycelial growth on all the target fungi tested. The isolates B27 and B36, which were fluorescent on TSA, were relatively weaker. In a similar manner, *Rhizoctonia* sp. was more sensitive to most of the antagonistic bacterial isolates, but the growth of *Pythium aphanidermatum* was less inhibited (for example, 23.8% of IR by B23 and 20.6% by B26).

Culture filtrates of 10 antagonistic bacterial isolates were tested against 14 species of pathogenic fungi. As shown in Table 4, culture filtrate from the isolates B6 and B23 were more effective at suppressing mycelial growth of the fungi tested.

To compare the antimicrobial activities, 6 isolates were tested against 7 microorganisms and the results are shown in Table 5. *Corynebacterium rathayi* was inhibited in general by most of the isolates, but *Erwinia carotovora* subsp. *carotovora* was not affected at all. Even though the isolates B6, B23, B27 and B36 did not show antibacterial activities, but their antagonistic activity was observed against to *Saccharomyces cerevisiae*.

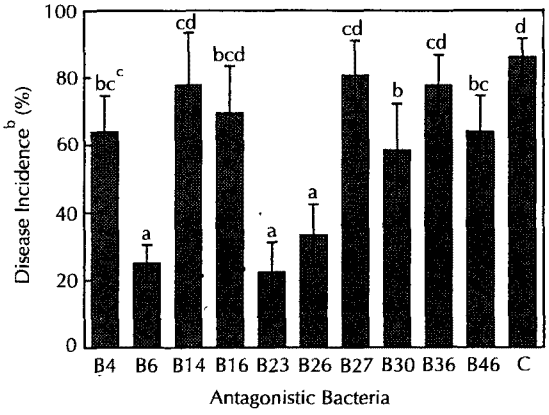

Fig. 1. Effect of cell-free culture filtrates of antagonistic bacteria on the disease incidence in sesame plants inoculated with *Fusarium oxysporum*[a].
[a]Spore suspensions (10^6/ml) of *Fusarium oxysporum* were prepared in 10% culture filtrates (in water), followed by drenching the soil in the sesame-growing pots.
[b]Disease incidence was rated 14 days after drenching the soil with *F. oxysporum*. Values represent means±standard deviations of 9 replicate plants.
[c]Values followed by the same letter are not significantly different at p =0.05.

A Greenhouse Test of Culture Filtrates

The cell-free culture filtrates from 10 bacterial isolates were examined in respect to controlling *F. oxysporum* in sesame plants grown in a greenhouse (see Fig. 1). The culture filtrates of the isolate B6, B23 and B26 reduced the infection of *F. oxysporum*, whereas the isolates B14, B27 and B36 exhibited no protective effect.

Identification of the Isolate B23

The isolate B23 was examined in accordance with procedures described in Bergey's Manual of Systematic Bacteriology, Laboratory Manual of General Bacteriology and the Analytical Profile Index System (API 50 CHB, bioMerieux, France), and the results in Table 6, 7 and 8 are shown in terms of the morphological, cultural, physiological and biochemical

Table 6. Morphological and cultural characteristics of the antibiotic-producing isolate B23.

Cell from	rod
Gram stain	+
Cell diameter > 1.0 μm	-
Spores round	-
Endospore produced	+
Motility	+
Anaerobic growth	-
Voges-Proskauer test	+
Growth in NaCl	
2%	+
5%	+
7%	+
10%	-
Growth at temperature	
5°C	+
10°C	+
30°C	+
40°C	+
50°C	-
55°C	-
Growth at pH 5.7, nutrient broth	+
pH 6.8	+

+; Positive, -; Negative.

Table 7. Physiological and biochemical characteristics of the antibiotic-producing isolate B23.

Catalase	+
Carbon utilization	
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Mannitol	+
Hydrolysis of	
Casein	+
Gelatin	+
Starch	+
Utilization of	
Citrate	-
Propionate	-
Degradation of tyrosine	-
Degradation of Phenylalanine	-
Nitrate reduction	+

+; Positive, -; Negative.

characteristics. B23 was finally identified as a strain of *Bacillus subtilis*.

DISCUSSION

In the rhizosphere of plants, diverse microorganisms interact with each other and this affects the growth and disease development of plants. Rhizospheres have been frequently exploited as excellent sources of biocontrol agents, since they may provide the front-line of defensive microroganisms for roots against the attack of soilborne pathogens (3, 5, 11, 20, 22).

In this study, we have isolated the antagonistic bacteria against *F. oxysporum* from the rhizosphere of sesame plants by the triple-agar-layer technique and evaluated their antagonistic activities. According to this technique, 327 isolates were selected from the 65 different soil collections. Among them, 83 isolates were determined to have antagonistic activity against *F. oxysporum*, and their inhibition rate varied depending on the type of media tested (TSA and PDA) suggesting medium composition as an important factor of consideration. The antifungal spectra of the selected isolates were also examined. In particular, the isolates B6 and B23 showed a broad range of antifungal activity. This broad antifugal spectrum has been reported from the studies of antagonists such as Actinomycetes, *Bacillus* sp., Pseudomonad and *Erwinia* sp. (8, 13, 17, 19, 21, 23, 28). The isolates B6 and B23 did not affect the growth of any target bacterial species tested in this experiment, but inhibited yeast. These characteristics would offer an advantage for the ecosystem in the plant rhizosphere by possibly causing no harm to the soil bacterial population.

The antifungal activity of cell-free culture filtrates of the bacterial isolates also were tested. Culture filtrates of isolate B6 and B23 strongly inhibited the mycelial growth of tested fungi, while ones from B27 and B36 showed no effect at all.

Little correlation exists between *in vitro* and *in vivo*

Table 8. Carbohydrate fermentation of the antibiotic-producing isolate B23 on the API 50 CHB.

Control	-	Glycerol	+	Erthritol	-	D-Arabinose	-	L-Arbinose	+
Ribose	+	D-Xylose	+	L-Xylose	-	Adonitol	-	β-Methyl-xyloside	-
Galactose	+	D-Glucose	+	D-Fructose	+	D-Mannose	+	L-Sorbose	-
Rhamnose	-	Dulcitol	-	Inositol	+	Mannitol	+	Sorbitol	+
α-Methyl-D-mannoside	-	α-Methyl-D-glucoside	+	N-Acetyl-glucosamine	-	Amygdaline	+	Arbutine	+
						Maltose	+	Lactose	+
Esculine	+	Salicine	+	Cellobiose	+	Inuline	-	Melezitose	-
Melibiose	+	Saccharose	+	Trehalose	+	Xylitol	-	β-Gentiobiose	+
D-Raffinose	+	Amidon	+	Glycogene	+	D-Fucose	-	L-Fucose	-
D-Turanose	+	D-Lyxose	-	D-Tagatose	-	2-ceto-gluconate	-	5-ceto-gluconate	-
D-Arabitol	-	S-Arabitol	-	Gluconate	+				

+; Utilized, -; Not utilized.

antagonistic activity in general (2). Identification of promising field-effective bacteria, however, can be faciliated by greenhouse experiments (27, 28). In our tests, some of the *in vitro*-selected bacteria (6 out of 10 isolates) efficiently suppressed the development of *Fusarium* in sesame plants when their culture filtrates were applied. In particular, the bacterial isolates B6 and B23, which exhibited antagonistic acivity with a broad antifungal spectrum *in vitro*, also showed suppressive effects in sesame plants at high level. However, no consistancy was observed in general during *in vitro* and *in vivo* experiments. For example, the isolate B30 did not suppress *F. oxysporum* infection *in vivo*, although the isolate showed antagonistic activity *in vitro*.

The isolate B23 was identified as *Bacillus subtilis*. The antibiotic substance from isolate B23 will be characterized to fulfull the need of developing a potential bioagent which controls not only Fusarium wilt in sesame plants, but also other plant fungal diseases.

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