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Bacillus subtilis YB-70 as a Biocontrol Agent of *Fusarium solani* causing Plant Root-Rot

KIM, YONG-SU, HO-SEONG LIM, AND SANG-DAL KIM*

Department of Applied Microbiology, Yeungnam University, Kyongsan 712-749, Korea

A bacterial strain YB-70 which has powerful biocontrol activity against *Fusarium solani* causing plant root-rot resulting in considerable losses of many economical crops was isolated and selected from over 500 isolates from a ginseng rhizosphere in suppressive soil, and identified as a strain of *Bacillus subtilis*. In several biochemical and *in vitro* antibiosis tests on *F. solani* with culture filterates from *B. subtilis* YB-70, our data strongly indicated metabolites which mediated inhibition of the fungal growth were presumed to be heat-stable, micromolecular, and ethyl alcohol solutable antifungal substances. Suppression of root-rot by *B. subtilis* YB-70 was demonstrated in pot trials with eggplant (*Solanum melongena* L) seedlings. Treatment of the seedling with the bacterial suspension ($1.7 \sim 1.9 \times 10^5$ CFU/g) in *F. solani*-infested soil significantly reduced disease incidences by 68 to 76% after 25 to 30 days. The results supported that *B. subtilis* YB-70 have excellent potentials as a biocontrol agent.

Biological control of soilborne plant pathogens by the addition of antagonistic microorganisms to the soil may offer a practical supplement or an alternative to existing disease management strategies that depend heavily on chemical pesticides (3, 6, 7, 9).

Agrochemicals for disease control are criticized severely for causing environmental pollution and residual problems (5), and consequently microbial disease control agents are expected to be safer and more economical for sustainable agriculture (20). The native method of reducing incidences of plant disease is being intensively studied and many ultimately augment or replace current chemical methods of control (7, 11, 12, 20).

There are two main ways of controlling phytopathogenic fungi causing the soilborne disease of agricultural crops: one is taking advantage of the antagonistic microorganism, and the other is an application of microbes to increase the host resistance (4). Antagonistic microorganisms, through their interactions with various soilborne plant pathogens, play a major role in microbial equilibrium and serve as powerful agents for biological control (3, 6, 9). These microorganisms have the potential for being superior biocontrol agents, and could be the most important factor in controlling plant disease and their ultimate being accepted in commercial disease management.

*Corresponding author
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Soilborne fungi cause some of the world's most important and destructive disease of plants, and for a number of them there are no effective chemical control methods (15). Root-rot, an major root disease of economically important crops caused by *Fusarium solani*, is widely distributed and responsible for nursery and field losses among a large variety of plants, especially ginseng (14, 20).

The objectives of this study were to isolate and identify the potentially useful bacterial antagonist from a suppressive soil, determine their chracterization of antifungal mechanism against *F. solani*, and evaluate the antifungal activiy both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Culture and Growth Medium

All isolates from the suppressive soil rhizosphere were grown on nutrient agar (NA) at 30°C. For the production of antifungal substances, antagonist was grown at 30°C for 3 days on a rotary shaker in a dextrose glutamate (DG) medium (pH 6.5) containing 1% dextrose, 0.5% DL-glutamic acid, 0.102% $MgSO_4$, 0.1% K_2HPO_4, 0.05% KCl, 4.4 μM $MnSO_4$, 9.6 μM $CuSO_4$, and 8.8 μM $FeSO_4$. Plant pathogenic fungus *Fusarium solani* causing root-rot of such important crops as ginseng was provided by Korea Ginseng and Tobacco Research Institute (KG-TRI) and maintained on a potato dextrose agar (PDA) at 28°C.

Isolation of Antagonist

Antagonistic bacteria were isolated from rhizospheres in ginseng root rot-suppressive soils in Yeungpung, Korea. Appropriate serial dilutions from soil suspensions in 0.01 M phosphate buffer (pH 7.2) were plated on NA and the plates were incubated at 30°C for 48 hr. Antagonistic activties of selected bacteria against *F. solani* were determined according to the antifungal tests described below.

Antifungal Tests

Antifungal tests against *F. solani* were assayed according to Lim *et al* (14). All bacterial isolates were initially screened for the ability to inhibit fungal growth with two different techniques. In the first assay, which tested the antifungal activity of bacterial strains on plates, samples (5 *µl*, containing approximately 10^6 cells) from the overnight cultures of bacterial strains in nutrient broth (NB) were inoculated around 1 cm from the edge of plates and allowed to soak into the agar. An agar disk (5 mm in diameter) of *F. solani* inoculum from the leading edge of a culture of *F. solani* grown at 28°C for 3 days on PDA was placed in the center of the plate. Plates were incubated at 28°C and scored after 5 days by measuring the distance between the edges of the bacterial colonies and fungal mycelium. In the second assay, which tested the antifungal activity in the broth culture, bacterial cultures were grown at 30°C for 84 hr with aeration. Cells were removed by centrifugation at 12,000×g for 20 min. The culture supernatants were then filtered aseptically through a membrane filter. Small plugs taken from 2 to 3-day-old cultures of *F. solani* which were then added to 250 m*l* Erlenmeyer flasks containing 2.64% potato dextrose broth (PDB) were incorporated aseptically with 5% culture supernatants and incubated on a rotary shaker at 28°C for 5 days. Fungal mycelia were collected on oven-dried preweighed paper (Whatman No. 2 filter paper) and dried at 105°C, and the dry weights were determined. The inhibition ratios were expressed relative to a control with water.

To isolate the most powerful antagonistic bacteria, two other methods were used in our laboratory. (i) Agar piece test. An agar disk of DG plates was cut with a sterilized corker bore (diameter 6 mm), and then were transferred into a new sterilized petridishes containing a moist filter paper (Whatman No. 2). Bacterial isolates were inoculated on the agar disks, and incubated at 30°C under about 100% relative humidity (RH). 2-day-old bacterial agar disks were patched along the perimeters of PDA plate on which 0.1 m*l* of a suspension of spores of *F. solani* (10^5 to 10^6 spores in H_2O) was placed in the center or spread over the entire surface of the plate. The plate was incubated at 28°C for 4 to 5 days, and the antifungal activity was determined by measuring zones of fungal growth inhibition. (ii) Agar diffusion test. Pertri plates were filled with molten PDA with autoclaved bacterial culture filterates. After the plates were cooled, an agar disk (5 mm in diameter) of *F. solani* inoculum grown at 28°C for 3 days on PDA was placed on the agar surface, and the plates were incubated at 28°C for 5 to 7 days. The diameters of the *F. solani* colonies were recorded, and the inhibition ratios were calculated relative to that of a control without incorporated culture filtrate.

Identification of Selected Bacterium

Identification of selected antagonistic strain was carried out according to the methods described in Bergey's Manual of Systematic Bacteriology (13) and Laboratory Manual of General Bacteriology (17).

Pot Bioassay

To determine the antifungal ability of the selected bacterium to protect plant against root-rot by *F. solani*, eggplant (*Solanum melongena* L) seedlings were used as the host plant in our study. Because they do not take a long time to germinate and maturate, the seedlings were good sources in root-rot bioassay. Eggplant seeds were germinated in a incubator at 30°C for 2 days at 100% RH, and thinned to uniform plants per polypropylene pot (25×25×55 cm). The bacterium tested for root-rot inhibition activity was applied to young plants with primary leaves one-fifth to one-third expanded because seedlings were attracted with root-rotting disease caused by *F. solani* rather than seeds. The bacterial suspension was prepared by bacterial growth in DG broth for 84 hr at 30°C with shaking at 150 rpm. The bacterial culture was then centrifuged at 10,000 rpm for 10 min and cells were resuspended in water. The desired concentration (approxmately 1×10^8/m*l*) was obtained by adjusting the suspension according to the standard curve with a spectrophotometer (Hitachi U 2000). Pots containing sterilized soil infested with *F. solani* (16~20 mg/g) were planted with 25 eggplant seedlings each, and then each seedling was covered with 5 m*l* of bacterial suspension prepared as described above. Pots were watered every other day with 500 m*l* each. To assess the disease incidences, the number of healthy eggplants was recorded and the percentage of roots with lesion induced by *F. solani* was estimated every 3 days. After 30 days, eggplants from the pots were recovered and examined to determine the level of disease.

RESULTS

Selection of Strains Inhibitory to Fungal Pathogen

For the selection of potential antagonists which inhibit to soilborne plant root-rotting *F. solani*, over 500 isolates

Table 1. Selelction of antagonistic bacteria against *F. solani* from rhizosphere in suppressive cultivated soil

Strains	Antifungal activity		
	Fungal dry wt. (%)[a]	Fungal colony size (%)[b]	Inhibition radius (mm)[c]
YB-70	40.2	51.6	20.8
YC-11	86.2	100.0	0.8
YK-25	66.7	100.0	2.5
YK-57	69.5	100.0	2.0
YK-88	47.9	53.8	19.3
YM-18	50.9	56.5	16.8
YM-29	64.7	84.7	11.4
YM-32	42.2	61.1	15.8
YS-13	45.1	88.7	10.9
YS-18	70.2	100.0	1.3
YS-26	63.1	99.7	6.9
YS-44	60.5	99.5	9.5
H_2O	100.0	100.0	—

The bacteria were grown in dextrose glutamate (DG) medium at 30°C for 48 hr.
[a]; Relative dry weight of *F. solani* cultured with bacterial culture filtrates to those cultured with water in potato dextrose broth (PDB) after 5 days of incubation at 28°C.
[b]; Relative colony circle size of *F. solani* cultured with autoclaved - bacterial culture filtrates to those cultured with water on potato dextrose agar (PDA) plates after 5 days of incubation at 28°C.
[c]; Distance between the edges of the bacterial agar pieces (about 5 mm square) and fungal mycelium on PDA plates after 5 days of incubation at 28°C.

of bacteria were initially obtained from root-rot suppressive soil in Yeungpung, Korea. Among these isolates, 70 isolates were investigated antifungal activities to *F. solani*. In primary screening, only 12 isolates produced inhibition zones of 15 mm or more with *F. solani* on PDA. From the results of the primary screening, antifungfal activity of twelve isolates were tested by cell mass method. To screen for isolation of more powerful antagonistic microorganisms, the isolates which showed interesting activities in the primary and secondary screening tests were grown in DG for 3 days at 30°C on the shaker. The culture filtrates were used to perform two preliminary experiments: agar piece test and agar diffusion test. These methods on plates inoculated with *F. solani* were used to measure antifungal substances production. The isolate YB-70 having the highest antifungal activity was selected among the twelve isolates and used in this study (Table 1).

Identification of the Isolate

The morphological, cultural, physiological, biochemical, and nutritional characteristics of the isolate YB-70 are presented in Table 2 and an electron micrograph is shown in Photo. 1. Staining and physiological tests showed that the strain was a Gram-positive, endospore forming, motile, aerobic, rod bacteria (Table 2). It all showed catalase, nitrate reduction, and VP, positive. It hydrolysed casein, gelatin, and starch but did not

Table 2. Morphological and culture characteristics of the isolated strain YB-70

Characteristics	Isolate YB-70
Cell form	rod
Cell diameter>1.0 μm	−
Gram strain	+
Strain Gram-positive at least in young cultures	+
Endospore produced	+
Spore round	−
Motility	+
Anaerobic growth	−
pH in Voges-Proskauer broth<6	+
>7	−
Growth at pH 6.8, nutrient broth	+
5.7	+
Growth in NaCl 2%	+
5	+
7	+
10	±
Growth at 5°C	−
10	+
30	+
40	+
50	+
55	−
65	−
Growth with lysozyme present	+

+: Positive, −: Negative, ±: Doubtful.

Table 3. Physiological, biochemical and nutritional chracteristics of the isolated strain YB-70

Characteristics	Isolate YB-70
Catalase test	+
Sulfate actively reduced to sulfide	+
Voges-Proskauer test	+
Acid from D-glucose	+
L-arabinose	+
D-mannitol	+
Gas from glucose	−
Hydrolysis of casein	+
gelatin	+
starch	+
Utilization of citrate	+
propionate	−
Degradation of tyrosine	−
Deamination of phenylalanine	−
Egg-yolk lecithinase	−
Nitrate reduced to nitrite	+
Formation of indole	−
dihydroxyacetone	±

+: Positive, −: Negative, ±: Doubtful.

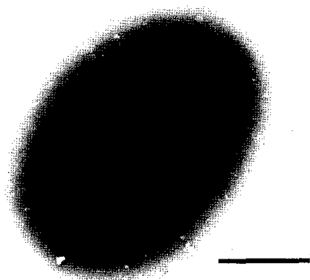

Fig. 1. Electron micrography of *B. subtilis* YB-70 producing endospore.
The bar denotes 1 μm.

produce indol and acid from glucose (Table 3). After due consideration of microbiological characteristics of the strain and reference to Bergey's Manual of Systematic Bacteriology and Laboratory Manual of General Bacteriology, the isolated YB-70 was identified as a strain of *Bacillus subtilis.*

Properties of Antifungal Substances

For the investigation of the properties of antifungal substance produced from selected *B. subtilis* YB-70 against *F. solani*, antifungal activities in the culture filterate of the strain were compared on the following three subjects: (i) dialyzed culture filterate on cellulose dialysis sack (MW 12,000), (ii) heat-treated culture filterate at 100°C for 30 min, and (iii) ethanol extracted solution from acidic (pH 2.0) preciptates of culture filterate. As shown in Table 3, after 5 days of incubation at 28°C, the culture filtrate inhibited growth of *F. solani* by 58.7%, whereas dialyzed solution inhibited growth by 10.3%. Loss of antifungal activity after treatment with dialyzed solution was 89.7%, compared with the activity of the culture filtrate. However, only 10.3 and 9.3% of the antifungal activity was lost when *F. solani* was treated with heat-treated and enthanol solutes, respectively.

For the practical assay of antifungal properties of *B. subtilis* YB-70, antifungal activities in the culture filtrate were compared by the following test. The cultural fitrate was separated by centrifugation at 2,500 rpm for 10 min through an Amicon centriprep 10 (No. 4304, molecular weight cut-off 10,000). Antifungal activity of each separate was determined by the cell mass method, and was expressed relative to a control (H_2O). After 7 days, the antifungal activity of culture filtrate against *F. solani* was 60.5%. Over 80%, compared with total activity, was due to the activty of micromolecules (<MW 10,000) (Fig. 2).

According to these results, antifungal substances in inhibitory mechanism of *B. subtilis* YB-70 against *F. solani* were presumed to be heat stable, micromolecular, and ethyl alcohol solutable substances.

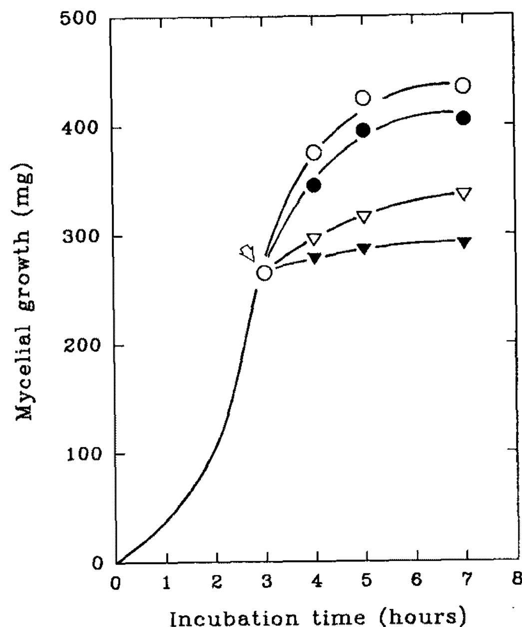

Fig. 2. Effect of antifungal substances from *B. subtilis* YB-70 on mycelial growth of *F. solani.*
3 day-old *F. solani* cultures were treated with antifungal substances from *B. subtilis* YB-70 grown in DG medium at 30°C for 84 hr. ○—○; Control (H_2O), ●—●; Macromolecular substance (>MW 10,000) of culture filterate, ▽—▽; Micromolecular substance (<MW 10,000) of culture filterate, ▼—▼; Culture filterate.

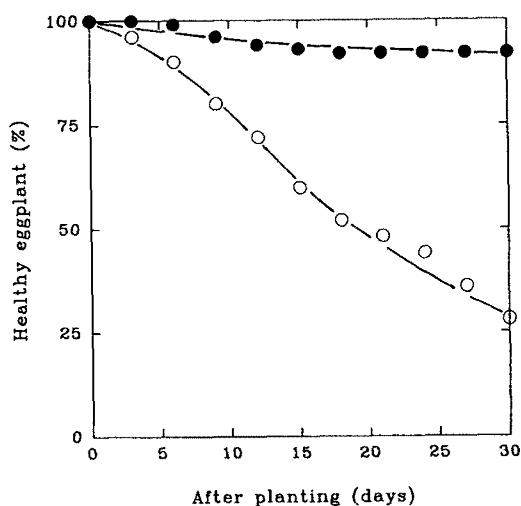

Fig. 3. Influence of *B. subtilis* YB-70 on the healty eggplant in soil infested with *F. solani.*
Polypropylene boxes (25×25×7 cm) were filled with the infested soil (16~20 mg/g of soil), planted bacterial suspension ($1.7\sim1.9\times10^5$ cfu/g) with 2 day-old seedling each and the number of diseased seedling recorded. All experiments were conducted under room condition of May. ○—○; *F. solani*, ●—●; *F. solani* with *B. subtilis* YB-70.

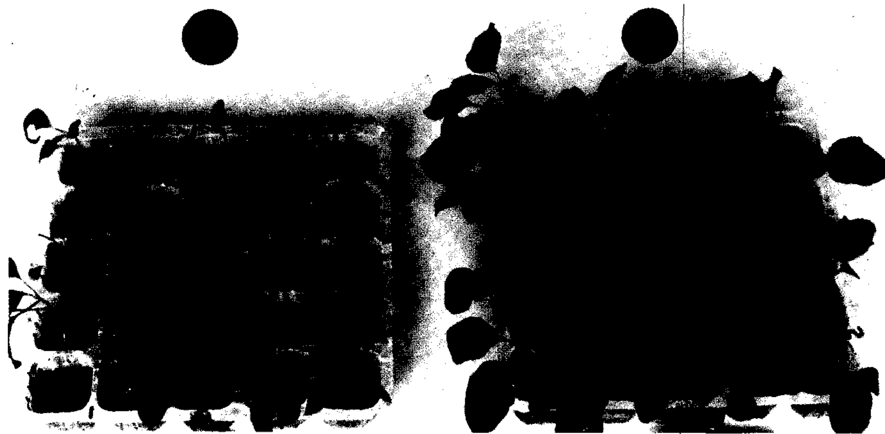

Fig. 4. *In vivo* suppressive effect of *B. subtilis* YB-70 against phytopathogenic *F. solani*.
A: *F. solani* only, B: *F. solani* with *B. subtilis* YB-70.

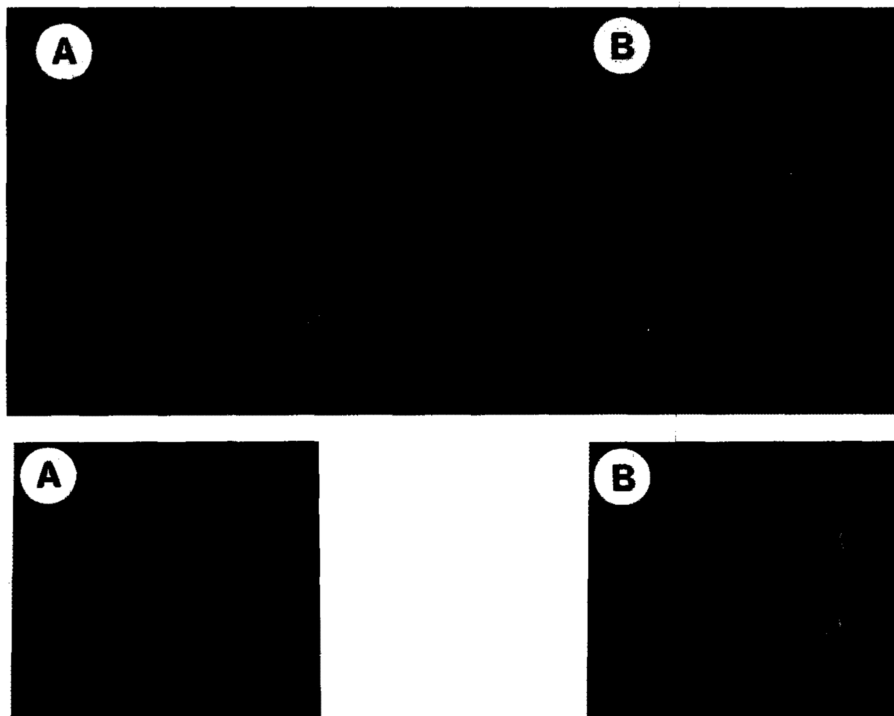

Fig. 5. Root-rot suppressive effect of *B. subtilis* YB-70 in rhizosphere soil.
A: *F. solani* with *B. subtilis* YB-70, B: *F. solani* only.

In vivo Biocontrol Test

Application of the antagonistic bacteria and pathogen to the rhizosphere confirmed that the bacteria which inhibited growth of the pathogen *in vitro* did the same on the rhizosphere and that biocontrol of root-rot lesions by bacteria was possible in a controlled environment (10). Hence, to evaluate the biocontrol ability of *B. subtilis* YB-70, the cells were treated in the pots containing eggplant. The percentages of healthy eggplants from seedlings transplanted in pot infested with *F. solani* are shown in Fig. 3. Application of *B. subtilis* YB-70 in soil infested with *F. solani* resulted in an 76% disease suppression of seedling root-rot on eggplant after 30 days of the treatment, whereas for the control, it was 24%. *B. subtilis* YB-70, the most inhibitory to fungal growth *in vitro* test, gave the most disease control rate in the pot test containing eggplant (Fig. 4). After 30 days, well-developed roots of eggplant in pot treated with *B. subtilis* YB-70 demonstrated the antagonist to have potential biocontrol ability against root-rot by *F. solani*

(Fig. 5).

DISCUSSION

Fusarium solani is a soilborne pathogen that causes root rot on many important vegetable crops. The use of microorganisms as a biological control for this phytopathogen is of interest because there is no chemical means to effectively control this fungus, and resistant cultivars are not available (12, 16). From this viewpoint, it is imperative to develop a new natural management for root-rot caused by this fungus. Therefore, major objective of this study were to select a powerful biological bacterium, and to estimate its potential ability against root-rot caused by *F. solani*. In our results, the most effective antagonist, a strain YB-70 was isolated from the ginseng rhizosphere, and identified it as a strain of *Bacillus subtilis* (Table 1, 2, 3 and Fig. 1). Selection procedures gave some indication of the mechanism of interaction between *B. subtilis* YB-70 and *F. solani*. The mechanism could be involve a antifungal substances rather than a lysing agent like an extracellular enzyme. This was confirmed by several biochemical tests with culture filtrates of *B. subtilis* YB-70 that the antifungal substances involved in the inhibition of *F. solani* appeared to be heat-stable, micromolecular, ethanol solutable substances (Table 4, and Fig. 2). Also, treatment of eggplant seedling with *B. subtilis* YB-70 facilitated the establishment of stands of healty eggplants in *F. solani*-infested soil (Fig. 3, Fig. 4, 5). Hence, the activity of the antagonist *in vitro* test was positively correlated to their activity against *F. solani in vivo* test. Other similar studies have proved correlations between the antibiotic production and the disease suppression (12). Similarly, in several studies on the previous use of *B. subtilis* for control of several diseases caused by plant pathogenic fungi (1, 2, 6, 15, 18, 19), the production of antibiotic substances by the strains has been recongnized as a major factor in the suppression of many root-rotting pathogens (1, 6, 15, 19). And they have suggested that this is probably due to the reduction of inoculum potential of the pathogen (11, 12). Although any definite evidence is still lacking, antibiosis in this study is regarded as an important mechanism in biological control (6, 15, 19).

Establishment and domination of antagonistic microorganisms in the infection court could be a prerequisite to the biological control (8). Effective induction and maintenance of disease suppression by introduced microbial antagonists may be largely dependent on the survival of these agents. (8). Thus, *B. subtilis* is a spore-forming bacterium that seems to survive well, at least

Table 4. Antifungal activity of the substances produced by *Bacillus subtilis* YB-70 against *F. solani*

Prepn	Fungal dry weight[e]		Fungal colony size[f]	
	Inhibition (%)[g]	Relative (%)	Inhibition (%)[h]	Relative (%)
Culture filterate[a]	59.5	100.0	57.9	100.0
Dialyzed sol.[b]	10.7	17.9	9.9	17.1
Heat-treated sol.[c]	49.4	83.2	47.7	82.4
Ethanol solutes[d]	50.6	85.0	48.3	83.4

[a] *B. subtilis* YB-70 was grown in dextrose glutamate (DG) medium at 30°C for 48 hr. Cells were removed by centrifugation at 12,000×g for 20 min. The culture supernatants were then filtered aseptically through a membrane filter.
[b] Culture filtrate was dialyzed at 4°C for 3 days through a cellulose dialysis sack (molecular weight cut-off, 12,000).
[c] Culture filtrate was heated at 100°C for 30 min.
[d] Culture filtrate was precipitated by the addition of c-HCl (final pH 2.0), and then the pellets were extracted with ethanol.
[e] Dry weight of *F. solani* with the treatment of *B. subtilis* YB-70 in PDB after 5 days of incubation at 28°C.
[f] Colony circle diameter of *F. solani* with the treatment of *B. subtilis* YB-70 on PDA plates after 5 days of incubation at 28°C.
[g] Completed inhibition ratio (100%)-dry weight of *F. solani* cultured with solutions relative to those cultured with water.
[h] Completed inhibition ratio (100%)-colony circle diameter of *F. solani* cultured with solutions relative to those cultured with water.

Table 5. Suppressiveness of *B. subtilis* YB-70 and the culture broth of this bacteria *in vitro* test

Phytopathogenic fungi	Inhibition zone by the cell (mm)[a]	Inhibition zone by the broth (mm)[b]
Alternaria kikuchiana	9	14
Alternaria mali	9	16
Botryis cinerea	15	11
Colletotrichum gloeosporioides	11	15
Collectrichum sp.	12	13
Fusarium oxysporum Schlecht	17	13
Fusarium oxysporum f.sp. *cucumerinum*	8	3
Fusarium solani	16	14
Gaeumannomyces graminis	30	32
Penicillium expansum	29	25
Phytophthora capsici	22	18
Pyricularia oryzae	18	20
Pythium ultimum	4	N[c]
Rhizoctonia solani	9	8

Plates were incubated at 24°C or 28°C and scored after 5 days by measuring the distance between the edges of the bacterial colony/well and fungal mycelium.
[a] Each 50 µl of cell suspension (1×10^7 ml) from overnight culture of *B. subtilis* YB-70 was inoculated 2 cm from the center of PDA plates and allowed to soak into the PDA. Fungal agar pieces (about 6 mm square) growing at 24°C or 28°C for 3 days on PDA were placed in the center of the plates.
[b] The culture broth (50 µl) of this bacteria filled in a well (6 mm diameter) made in the center of PDA plates which were seeded with fungal spore suspensions (1×10^5 conidia/ml).
[c] No inhibitory zone.
Each value represents the mean of three plates.

in the soil (2, 18). *B. subtilis* YB-70 isolated in our laboratory produced antifungal antibiotics and exhibited a wide suppressive spectrum to various phytopathogenic fungi (Table 5). In this regard, *B. subtilis* YB-70 would seem to be a good candidate to a successful antagonist although questions remain as to the efficacy of *B. subtilis* YB-70 under field conditions. If the antifungal antibiotics are identified and the mode of action is well elucidated, the compounds will have the potential to control other plant diseases as well as root-rot.

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