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Effective Screening of Antagonist for the Biological Control of Soilborne Infectious Disease (Damping-Off)

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Abstract An efficient method of selecting an antagonistic strain for use as a biological control agent strain was developed. In this improved method, the surface tension reduction potential of an isolate was included in the 'decision factor,' in addition to two other factors; the growth rate and pathogen inhibition. By using a statistically designed method, an isolate from the soil was selected and identified as *Bacillus* sp. GB16. In the pot test, this strain showed the best performance among the isolated strains. The lowest disease incidence rate and fastest seed growth were observed when the *Bacillus* sp. GB16 was used. The action of the surface tension reducing component was assumed to enhance the wetting, spreading, and residing of the antagonistic strain in the rhizosphere. This result showed that the improved selection method was quite effective in selecting the best antagonistic strain for the biological control of soilborne infectious plant pathogens.

Key words: Biological control, damping-off, antagonist, biosurfactants, relative performance index

The excessive use of chemical pesticides induces the occurrence of resistant microorganisms [52] and reduces agricultural production potentials due to acidification of the soil and decrease of indigenous antagonists in the soil [10]. In particular, soil-borne infectious diseases are difficult to control by chemical pesticides, because they can be drained from the soil by rains. In addition, many other side effects can appear, such as the promotion of eutrophication and bioaccumulation [9, 44].

Therefore, there is increasing need for a biological control agent to produce nonpolluting crops [4, 57]. In addition, the registration of the commodity of the biological control agent is relatively easy for the chemical pesticide due to the safety to non-target living organisms and environmental suitability [39]. Many studies have presented a variety of biological control agents [17, 25, 26].

Damping-off is a disease that can affect almost all vegetable plants at the earliest stages of growth. It occurs in home gardens as well as in commercial fields and greenhouses, and may be caused by several soilborne fungi. The major fungi responsible for damping-off disease in plant nurseries, including *R. solani* Kühn and *Pythium* sp., have been controlled by several bacterial isolates [19, 21] and binucleate *Rhizoctonia* [18, 20] in vegetable and ornamental seedlings grown in a pasteurized potting medium.

Previous methods used for screening antagonists were to select a strain showing the highest growth inhibition of a particular plant pathogen. However, such isolate was not always the most effective antagonist in the field test [12, 37, 48, 51, 54]. The screening method, therefore, should take into consideration the field conditions such as microorganism's growth, motility, and attachment to the plant [25, 38, 43].

Bacilli, as the major microorganisms in biological control, produce the lipopeptide-biosurfactant that has antifungal and hemolytic activities [40, 41] and forms a stable endospore, which can sustain the control and/or suppression of soilborne infectious plant pathogens [15, 47]. Currently, surfactants are generally added to chemical pesticides in order to emulsify the pesticide and to increase the attachment to plants [7, 55].

In this study, we screened 87 strains isolated from healthy soils and composts, based on the two-dimensional selection RPI (Relative Performance Indices) method [54], considering the following three factors; (1) antagonistic activity against a plant pathogen, (2) growth rate, and (3) surface tension reduction. The antagonist strain, which was screened using this improved method, showed the best performance in the pot-test, indicating the effectiveness of the new screening method. In addition, we carried out chemical and physical mutations of finally selected strain to obtain a high

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hemolytic mutant, corresponding to lipopeptide production [40, 41].

MATERIALS AND METHODS

Antagonist Screening and Cultivation

Microorganisms antagonistic to *Pythium aphanidermatum* and *Rhizoctonia solani* were isolated according to the modified Herr's method [24]. Soil samples were collected from the rhizosphere 10 to 15 cm beneath the soil surface from the crop-cultivating fields at different locations in Gyeonggi-do, Korea. The compost samples were collected from several regions. The samples were stored at 4°C in plastic bags until needed.

Eighty-seven strains (#1–#87) isolated from the various composts and healthy soils were used as candidates for the antagonist. *Bacillus subtilis* ATCC21332 [Kodiak; (Gustafson, Texas)] producing surfactin and *Bacillus* sp. H6 [16] that was previously isolated by our laboratory were used as the positive control. The screening of the antagonist was based on the abilities to inhibit pathogens, the growth rate measured by the viable cell number, and the surface tension reduction by introducing the RPI to the ranking strains [54].

i) $RPI_{efficacy}$ for antagonistic activity

$$\left|\{(x_1 - \bar{x}_1)/(\sigma - 2)\}\right| \times 25$$

x_1; single observation value for a strain

$\bar{x}_1$; average of all observations from all strains being ranked

σ; standard deviation of all observations from all strains being ranked

ii) $RPI_{kinetics}$ for viable cell number

$$\left|\{(x_3 - \bar{x}_3)/(\sigma+2)\}\right| \times 25$$

x_3; single observation value of viable cell number for a strain

iii) $RPI_{surface}$ for surface tension reduction

$$\left|\{(x_4 - \bar{x}_4)/(\sigma+2)\}\right| \times 25$$

x_4; single observation value of surface tension reduction for a strain

The efficacy was calculated by measuring inhibition rate of dual culture pathogen and candidates on PDA (Potato Dextrose Agar) plate.

inhibition rate=(control−experiment)/control×100

Mycelia plugs (5-mm diameter) collected from the margin of the actively growing hypha of *P. aphanidermatum* and *R. solani* were placed 5 and 4 cm apart from the candidates on the surface of the PDA medium and allowed to grow at 25°C. The *P. aphanidermatum* and *R. solani* plates were cultivated for 2 and 3 days, respectively.

The surface tension of the cell-free culture broth was measured at room temperature by du Nouy tensiometer. (Fisher Surface Tensiomat, Model 21, Fisher Scientific, Pittsburgh, PA, U.S.A.).

The antagonistic candidates were cultivated (250 rpm, 30°C, 16 h) in a 15-ml test tube containing 5 ml LBS medium (soluble starch, 10 g/l; tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 5 g/l) and subsequently cultivated in a 30-ml test tube containing 10 ml of LBS medium (250 rpm, 30°C, 12 h).

The selected strain was cultivated at different initial pHs, 3, 5, 7, 9, and 11. The viable cell number and the final pH were measured to examine the effect of the initial pH on cell growth. The selected strain was cultivated at different temperatures, 25, 30, 37, and 45°C, and also cultivated in different working volumes in a 100-ml Erlenmeyer flask for measuring the oxygen requirement [35]. After cultivation, the number of viable cells and the final pH were measured.

The samples were withdrawn aseptically to analyze the number of viable cells and spores. The colony-forming unit (cfu) was measured using a serial plating technique on LBS agar. The appropriately diluted samples were plated and incubated for 12 h at 37°C. For spore counting, appropriately diluted samples were treated at 80°C for 15 min prior to plating.

Identification

The selected strain #16 was stained by the basic Gram method [49], and was analyzed using an optical microscope. Catalase activity was determined in the supernatant and cell extract at room temperature using hydrogen peroxide as the substrate, by the method of Beer and Sizer [5]. Lipase activity was determined by measuring the free fatty acid produced from olive oil solution [27]. Urease activity was measured by quantifying the ammonia released from urea by formation of indophenol, which was measured at 625 nm [56].

Bacterial genomic DNA was prepared by a previously described method [32]. PCR amplification and DNA sequencing of the 16S rRNA gene were performed according to the Laha and Luthy method [33]. The DNA sequence was analyzed using a DNA sequencing system (ABI Prism 377 DNA sequencer, PE Applied Biosystems, Foster City, CA, U.S.A.). The sequence was aligned with other known 16S rRNA gene sequences of bacilli by using the PHYDIT version 3.1 (http://plaza.snu.ac.kr/~jchun/phydit/). The tree topology based on these aligned 16S rRNA gene sequences was reconstructed with the Kimura's two-parameter distance model [29] and neighbor-joining methods by the PHYLIP 3.5 package [13].

Mutation

The cells in the logarithmic growth phase were collected by centrifugation (10,000 ×*g*, 10 min, 4°C). The washed cell pellet was resuspended in saline containing 250 μg/ml *N*-

methyl-*N*-nitro-*N*-nitroso-guanidine (NTG) (Sigma Chemicals, St. Louis, MO, U.S.A.) at a concentration of 10^8 cells/ml. The suspension was shaken for 30 min at 37°C and the cells were harvested by centrifugation at 8,000 ×*g* for 10 min, washed twice with saline, and suspended in the same saline solution [53]. An aliquot (0.1 ml) of the suspension was spread on a LBS agar plates and the colonies were isolated after 2–3 days of incubation. The survival ratio was less than 1%. The ultraviolet mutation method was also used to obtain a high hemolytic mutant, corresponding to lipopeptide production. The cells in the logarithmic growth phase were washed with physiological saline and resuspended in saline. An aliquot (0.1 ml) of the suspension was spread on an LBS agar plate. Colonies that appeared during 12 h of incubation under a UV lamp (40 W, under 30 cm) were isolated.

Colonies possessing hemolytic activity were screened on a sheep blood agar plate used to identify lipopeptide production [34, 40].

Tolerance to Chemical Pesticides

The tolerance of the selected strain to 8 chemical pesticides (wettable powder) was measured by a diffusion method. These pesticides have been used to control damping-off, brown patch, and pythium bright caused by *P. aphanidermatum* and *R. solani*. Each chemical pesticide (50 µl) was introduced into autoclaved stainless steel cups (diameter 7 mm) on a lawn-cultured LBS agar plate with each selected strain. After cultivating at 30°C for 24 h, the tolerance of the selected strain to chemical pesticides was determined by measuring the diameter of the halo zone including the cup diameter.

Pot Test

P. aphanidermatum and *R. solani* hypha were cultured on PDA (5 days) and 10% Campbell's V8 juice agar (7 days), respectively. The samples were mixed with 100 ml of deionized water in a laboratory blender (Waring, U.S.A.) and blended for 1 min at full speed to suspend the hypha. The suspension was appropriately inoculated into the pot. The efficiency of the chemical pesticide and the biological control agent were calculated by comparing them with an Anta emulsion™ (etridiazole, 25% v/v, Dongbu Hannong Chem, Seoul, Korea) as the control chemical pesticide. Disease incidence was calculated by dividing the number of diseased seedlings by the total number of seedlings. The control effect of the test materials on damping-off was evaluated with control value (CV) calculated by the formula CV (%)=[(A−B)/A]×100, where A represents the number of infected seeds and seedlings in untreated pots, and B represents the number of infected seeds and seedlings in treated plants. Furthermore, seedling shoot height, shoot dry weight, root length, and root dry weight per pot were measured [1].

Results and Discussion

Inhibition of Fungal Growth

The inhibition rate was calculated by measuring the hyphal length of *P. aphanidermatum*, *R. solani*, and the candidate strains. The control plate was prepared by growing *P. aphanidermatum* and *R. solani* without the candidate strain. The inhibition rate against the rapidly growing *P. aphanidermatum* was low, and the most effective strains were #2 and #24, which showed a 27.1% inhibition rate. The most effective strain against *R. solani* was #42, and the inhibition rate was 51.5% (Fig. 1).

Antagonist Screening

Considering the growth of the candidate in antagonist screening, cell growth was generally measured using optical density, because of easy determination. However, the increase of optical density was not proportional to the increase of the viable cell number due to difference in the cell size and the presence of extracellular products such as pigments

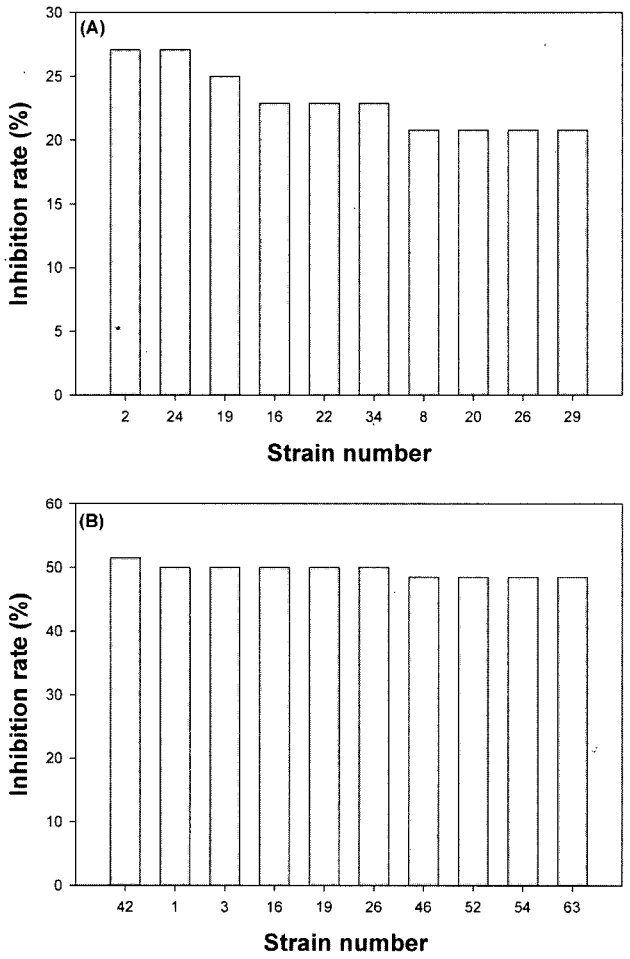

Fig. 1. Inhibition rate of the candidate strains against *P. aphanidermatum* (A) and *R. solani* (B).

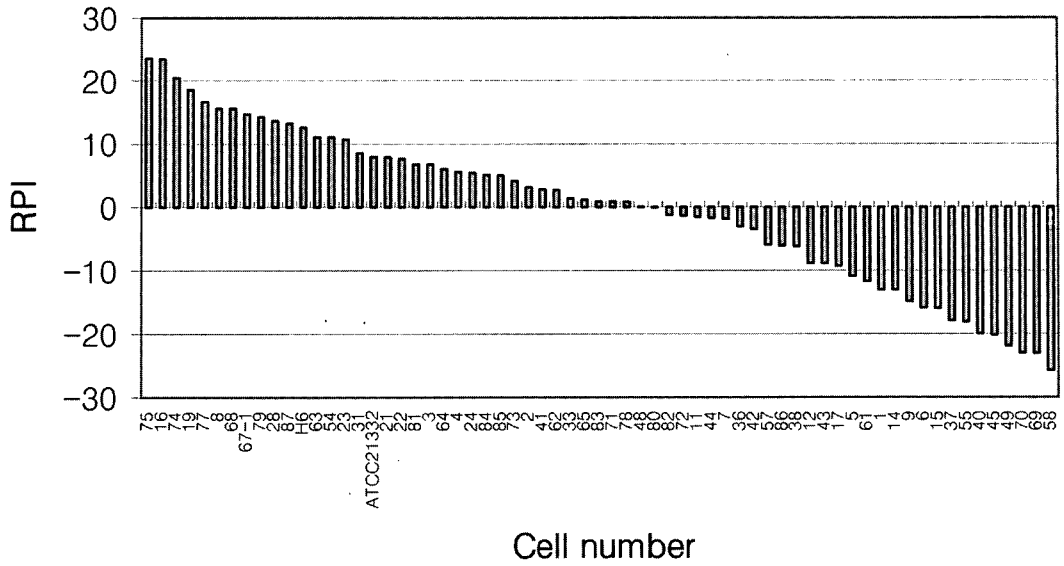

Fig. 2. Use of the relative performance indices (RPI, averages of $RPI_{efficacy}$, $RPI_{kinetics}$, and $RPI_{surface}$ values) to achieve two-dimensional assessment of antagonistic organisms based on growth (total cell number), the efficacy of the cells, and surface tension reduction.

and the properties of the cell surface. Therefore, cell growth was measured using the viable cell count in antagonist screening, [23, 50].

Two strains, #75 and #16, were selected by comparing 4 RPI averages of the cell growth and inhibition rate to *P. aphanidermatum* and *R. solani* as well as surface tension reduction (Fig. 2). Twenty-two strains of candidates were excluded from further studies due to their low cell growth on LBS media and/or PDA plate. After cultivating the #75 and #16 strains, #16 was finally selected by the stability of the viable cells (Fig. 3). Strain #75 was superior to #16 only in the cell growth (test tube culture), but the other RPI values were low.

Previous methods for screening of antagonist using the inhibition rate *in vitro* only resulted in a discrepancy with the control efficiency *in situ* or often a depreciation due to the slow growth rate, and was found to be improper as a commercial product [12, 37, 48, 51, 54]. Consequently, not only should the inhibition rate and growth rate of the candidate strain be considered, but also the factors of attachment to plant and the motility of candidates in the soil condition [25, 38, 43, 44]. Generally, a chemical surfactant is added to the chemical pesticides that have been used to control the plant pathogen, to emulsify the pesticide and increase the attachment to the plant [7, 55].

When the surface tension reduction by the biosurfactant in antagonist screening was considered, the antifungal activity, wettability-enhancing attachment on the solid surface, and the enhancement of the microbe screening were expected [2, 15, 25, 26, 28, 38, 42, 43, 45].

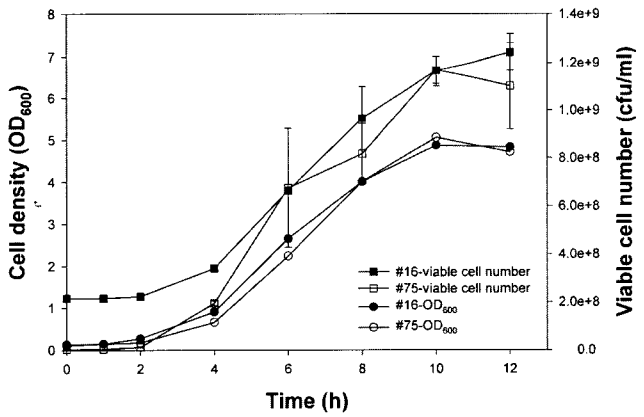

Fig. 3. Time course of cultivation of the isolated strains (#16, #75). Bar represents standard deviation.

Identification of Selected Strain

The selected strain #16 was Gram-positive, rod-shaped, endospore forming, and catalase, lipase, and urease positive (data not shown). The sequence of the 16S rDNA extracted from the selected strain was compared with that in GenBank and EMBL (European Molecular Biology Laboratory). The 1,496 base sequence in 16S rDNA was analyzed and compared to that of the *Bacillus subtilis* subsp. *subtilis* DSM10. It showed a 99.3% identity (Fig. 4). Therefore, the selected strain was designated as *Bacillus* sp. GB16 (GenBank accession number AY911607).

Determination of Optimal pH, Temperature, and Oxygen Need

Bacillus sp. GB16 did not grow at pHs 3.0 and 11.0, and the lag phase increased up to 10 h at pH 5.0, while the cell

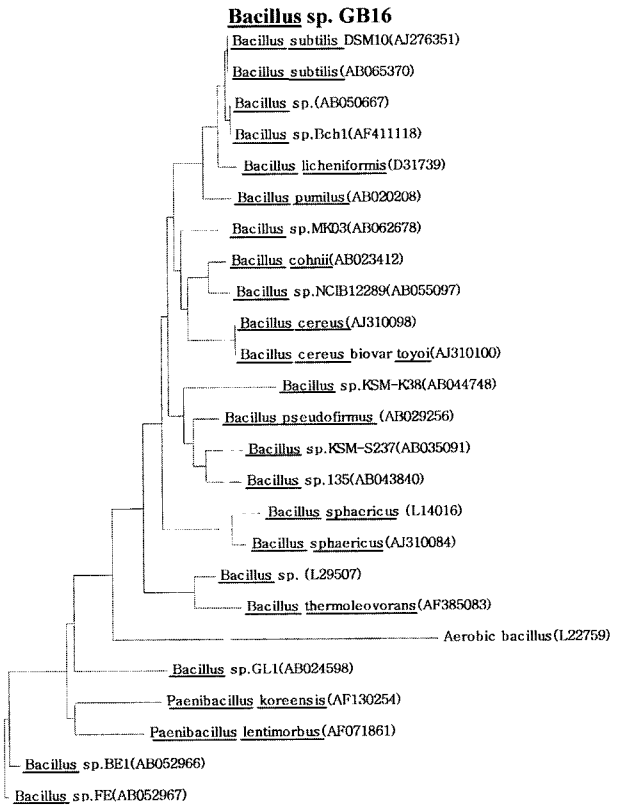

Fig. 4. A 16S rRNA tree constructed by the pairwise distance measures using the neighbor-joining method.

grew well at pHs 7.0 and 9.0. Optical density at pH 9.0 was superior to pH 7.0, although the lag phase was longer than those measured at pH 7.0. In addition, no cell lyses occurred at pH 9.0 (Fig. 5). Thus, the highest viable cell number was observed at pH 9.0 after 10 h of cultivation (data not shown). After 14 h of cultivation at pHs 5.0, 7.0 and 9.0, the final pHs were changed to pHs 6.1, 7.7, and 8.2,

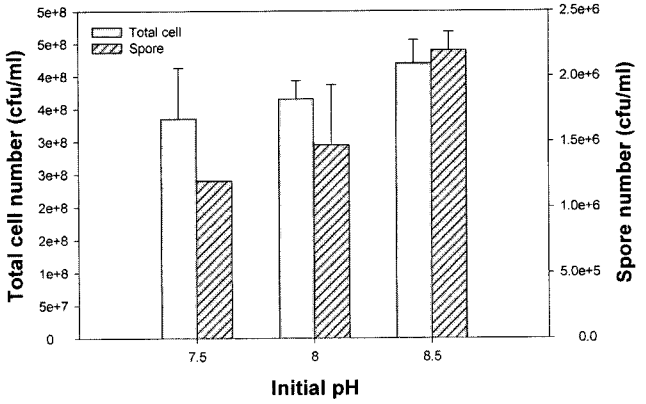

Fig. 6. Effect of initial pH on the total number of cells and spores at 12 h.
Bar represents standard deviation.

respectively. The pHs of acidic and neutral environments were increased while basic pH was decreased. Therefore, the experiments were carried out within a narrow range of pH values; the initial pH was adjusted with 3 N NaOH to pHs 7.5, 8.0, and 8.5. After 12 h of cultivation at pHs 7.5, 8.0, and 8.5, the final pHs were changed to pHs 8.0, 7.8, and 7.7, respectively. The cell growth at pH 8.5 was slightly retarded, compared with pHs 7.5 and 8.0, like pH 9.0. The highest cell growth was found at pH 7.5. Serious cell lyses was, however, observed after 8 h of cultivation (data not shown). After 12 h of cultivation, the culture at pH 8.5 showed a higher number of viable cells and spores due to less cell lyses (Fig. 6). Together, two results of the pH experiment showed that *Bacillus* sp. GB16 is a facultative alkaliphile. In order to determine the optimal temperature for cell growth, *Bacillus* sp. GB16 was cultivated at various temperatures. The cell growth and sporulation ratio were high at 37°C and there was no cell lyses. Cell growth was retarded at 25°C and 30°C and exhibited dramatic cell lyses

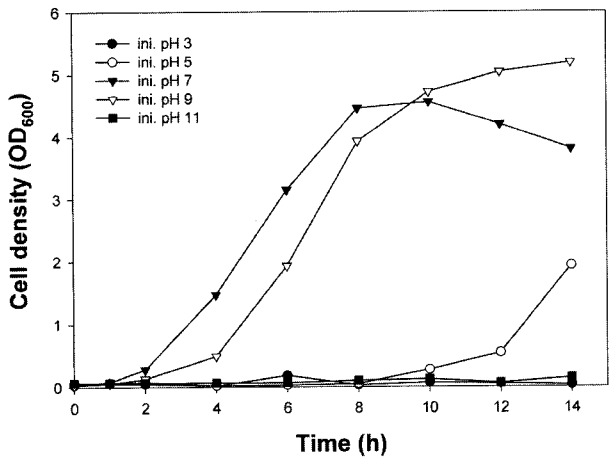

Fig. 5. Effect of initial pH on the cell growth.

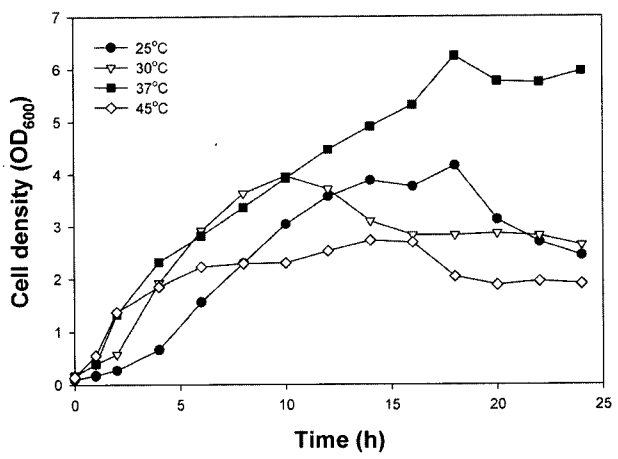

Fig. 7. Effect of temperature on cell growth.

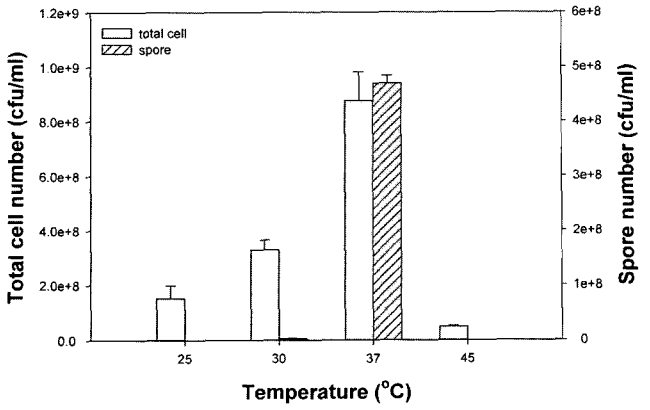

Fig. 8. Effect of temperature on cell growth and sporulation at 24 h of culture.
Bar represents standard deviation.

after a stationary phase. In addition, the spores were rarely formed at 25°C and 45°C (Fig. 7, Fig. 8). Figure 9 shows that *Bacillus* sp. GB16 required a high level of oxygen for better cell growth. The final pHs decreased with increasing working volume. The final pH declined to 6.5 after 12 h of cultivation with a 100 ml working volume (data not shown). It appears that organic acids were formed by insufficient aeration [22, 46].

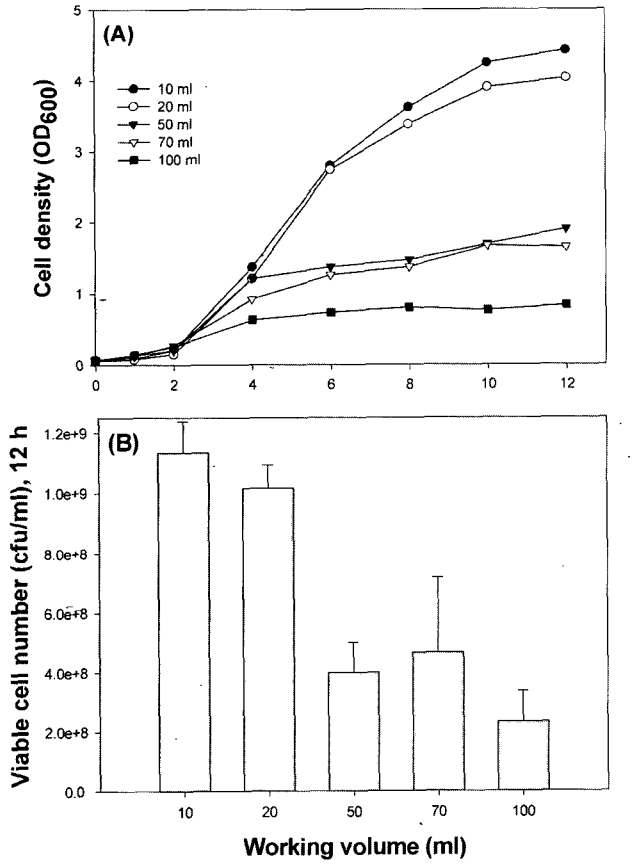

Fig. 9. Effect of working volume on cell growth (A) and viable cell number at 12 h of culture (B).
Bar represents standard deviation.

Relationship Between Hemolytic Activity and Antifungal Activity

Some nonhemolytic mutants (fully or slightly negative strains, 11 colonies from 500 colonies) were obtained by the NTG treatment. The nonhemolytic mutants not only formed tiny colonies on LBS agar plate, compared with wild-type and enhanced-hemolytic mutants, but could not form spore and did not exhibit antifungal activity (data not shown). The purified surface-active agent of *Bacillus* sp. GB16 had antifungal and hemolytic activities (data not shown). Therefore, the production of hemolytic substance is one of the most important factors that could confer antifungal activity to *Bacillus* sp. GB16 [1, 40, 41]. Unfortunately, we did not obtain nonhemolytic mutants from UV-resistant colonies.

Tolerance to Chemical Pesticides

The co-utilization of biological control agents and chemical pesticides may be a practical method in the field [31, 36]. *Bacillus* sp. GB16 was more tolerant to the existing chemical pesticides that have been used to control damping-off and grass turf caused by *P. aphanidermatum* and *R. solani* and contains iprodione, etridiazole, and metalaxyl. However, *Bacillus* sp. GB16 was quite sensitive to chemical pesticides containing bitertanol and dichlofluanid as the main components (Table 1) [6, 8, 11]. Therefore, *Bacillus* sp. GB16 could be co-utilized and introduced to soil treated with chemical pesticides containing iprodione, etridiazole, and metalaxyl.

Pot Test

The control values of the #16 and #1 strains for the damping-off caused by *R. solani* were superior to those of

Table 1. Resistance of *Bacillus* sp. GB16 to various chemical pesticides.

Pesticides (main components)	Pesticide concentration (mg/l)				
	0	2	20	200	2000
	Halo zone diameter (mm)				
Iprodione[a]	8	8	10	9	10
Etridiazole	9	12	10	7	11
Bitertanol	9	10	11	15	15
Thiram	7	7	9	13	13
Oxine-copper+iprodione[b]	8	7	11	10	13
Metalaxyl	7	7	8	12	10
Dichlofluanid	9	13	13	14	18
Iprodione[c]	7	8	9	10	11

[a]Robral™ (Dongbu Hannong Chemical, Seoul, Korea).
[b]Robdong™ (Dongyang Chemical, Incheon, Korea).
[c]Ipro™ (Yooill, Seoul, Korea).

Table 2. Disease incidence and control value of bacterial strains used for the biocontrol of damping-off.

Bacterial strain	*R. solani*		*P. aphanidermatum*	
	Disease incidence (%)	Control value (%)	Disease incidence (%)	Control value (%)
#1^{R2}	52	35	8	33
#2^{P1}	64	20	4	33
#3^{R3}	80	0	-	-
#16$^{P4, R2, S10}$	48	40	8	67
#19$^{P3, R2}$	80	0	8	33
#22^{P4}	64	20	4	67
#24^{P1}	76	5	8	33
#42$^{R1, G6}$	76	5	8	33
Etridiazole, EW	44	45	8	33
Control	80	-	12	-

P1, 1st strain of inhibition rate against *P. aphanidermatum*; R1, 1st strain of inhibition rate against *R. solani*; G6, 6th strain of cell growth (OD_{600}); S10, 10th strain of surface tension reduction; Control, non-treated seeds.
-, not detected.

the other strains (40% and 35%, respectively). The control values of #16 and #22 strain for the damping-off caused by *P. aphanidermatum* were 67%, which were superior to the other strains (Table 2). The results of the *in vitro* and *in vivo* assays were correlated; however, #2 and #42, which had the highest inhibition rate against *P. aphanidermatum* and *R. solani*, showed a low control value. These results confirmed that *in vitro* antagonistic assays in most cases are insufficient for establishing the biological control activity of a strain against the pathogens by considering only the inhibition rate. Several bacterial characteristics, such as rapid root adhesion, competition for colonization sites and nutrients [14], as well as some soil properties, will influence the antagonistic response [58]. In addition, bacteria under natural conditions could produce compounds involved in fungal inhibition that are not produced in the culture media [3].

When a surfactant is produced in the soil, it distributes itself between the solid-liquid interfaces. The solid-liquid interface consists of either *Bacillus* sp. GB16 and water or soil and water, and adsorption to the solid phase occurs in both cases [38].

The #16 and #19 strains accelerated the growth of roots in the pot infected with *R. solani*, while the #2 and #16 strains accelerated the growth of roots in the pot infected with *P. aphanidermatum* (Table 3). These results show that the selected strain is a plant growth-promoting rhizobacteria [30] as well as a biological control agent for the soilborne infectious disease, damping-off.

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Table 3. Effect of bacteria strains on cucumber seedling growth.

Bacterial strain	*R. solani*				*P. aphanidermatum*			
	Length (mm)		Weight (g)		Length (mm)		Weight (g)	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
#2	-	-	-	-	71	101	1.56	0.58
#16	92	86	1.76	0.41	96	92	2.19	0.76
#19	72	95	1.34	0.71	96	79	2.36	0.59
#42	88	79	1.54	0.35	-	-	-	-
Control	72	92	1.34	0.46	99	81	2.48	0.47

-, not detected.

strains capable of growth on organochlorine pesticides. *Bioresource Technol.* **89:** 133–138.
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