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Biological Control of Postharvest Root Rots of Ginseng

Hoo-Sup Chung*, Eun Sun Chung and Yong-Hwan Lee
Department of Agricultural Biology and Research Center for New Bio-materials in Agriculture, Seoul National University, Suwon 441-744, Korea

수확 후 인삼뿌리썩음병의 생물학적 방제

정후섭* · 정은선 · 이용환
서울대학교 응용생물화학부 및 농업생물신소재연구센터

ABSTRACT: The production of Korean ginseng, one of the most important medicinal root crops, is limited by many factors including soil sickness, root rots in fields as well as during storage prior to consumption. Although much research has been conducted on the diseases in field condition, little information is available on the control of postharvest roots rots. To obtain better management strategy of postharvest root rots in ginseng, biological control using antagonistic bacteria was attempted. Of 208 bacteria obtained from suppressive soil samples, 4 were selected based on the inhibitory effect on mycelial growth of two major causal fungi for postharvest root rots in ginseng, *Botrytis cinerea* and *Fusarium solani*. The culture filtrates of these bacterial antagonists greatly inhibited the conidial germination of both pathogenic fungi and produced abnormal morphology such as swollen germ tubes in *F. solani* and vacuolation of nongerminated conidia in *B. cinerea*. The population levels of bacterial antagonists on the ginseng roots were gradually increased up to 8 days of incubation. Postharvest root rots of ginseng caused by *F. solani* and *B. cinerea* were controlled in dipping tests in the ranges of 60~80% by antagonistic *Bacillus* spp. obtained from suppressive soil. These results suggest that biological control using these antagonistic bacteria would be an alternative strategy to control postharvest root rots in ginseng.

Key words: **biocontrol, *Panax ginseng*, *Botrytis cinerea*, *Fusarium solani***

Korean ginseng (*Panax ginseng* Meyer) has been recognized in the Orient as one of the most important medicinal root crops belonging to the Araliaceae family and its demand increases yearly. However, stable production of ginseng is limited by many factors including root rots caused by *Cylindrocarpon destructans* and *Fusarium solani* and postharvest diseases. Since ginseng requires fertile soil, rich in humus and with continuous shade for six years to grow until harvest, there is no practical control measure for root diseases in fields (4-6).

Postharvest diseases in fruits, vegetables, and root crops have not received the attention that the magnitude of the problem warrants, although they cause major losses in food production. In United States, it has been estimated that approximately 24% of harvested fruit and vegetables is lost to postharvest spoilage (18). Postharvest losses are even greater in developing countries where sanitation and refrigeration are lacking or minimal. Although the accurate estimation is not available, postharvest loss of ginseng may be equivalent to other fruits and vegetables. Root rots caused by *Botrytis cinerea* and *F. solani* account for the majority of postharvest loss in ginseng (4-6). Fungicide application is the primary means to control diseases in the field and postharvest stages. However, the residual toxicity of chemicals on foods and appearance of resistant strains of pathogens to chemicals require the alternative strategies for stable production and storage of ginseng (7).

Biological control of ginseng root rots caused by *C. destructans* and *F. solani* with nonpolluting soil amendments and antagonistic microorganisms has been tried during the past (5, 6). However, no attempt has been made on biological control of ginseng root rots during postharvest stages. Biological control of postharvest diseases is relatively new field, but there have been several encouraging developments (19). Much of this work has been concerned with the control of wound pathogens, such as *B. cinerea* (12, 15) and *Monilinia fructicola* (13). There are several advantages in using bio-

*Corresponding author.

logical control agents at the postharvest stage, because wounding of fruits and roots occurs mainly during harvesting and postharvest handling. Thus the antagonists can be applied when the wound is most likely to occur, and subsequently can occupy the wound surface in advance and prevent subsequent invasion by pathogens. Antagonistic bacteria and yeast, such as *Bacillus subtilis* (13, 14), *Pseudomonas cepacia* (10), *Enterobacter aerogenes* (16), and *Debaryomyces hansenii* (2, 3), have been tested to control postharvest diseases in a variety of stored vegetables and fruits including apple, peach, pear, cherry, potato, and tomato.

In this paper we have assessed the potential of biological control approach for the reduction of postharvest development of root rots in ginseng. Potential antagonistic bacteria were isolated from the suppressive soil, screened for antagonistic activity, and then tested for the control of root rots caused by *B. cinerea* and *F. solani*.

MATERIALS AND METHODS

Fungal strains and cultural conditions. *Botrytis cinerea* and *Fusarium solani* were isolated from decayed tissues of ginseng in the storage condition. These isolates were single-spored and maintained on potato carrot agar. Conidia of *B. cinerea* and *F. solani* were harvested from 5 and 8 days old plates, respectively, and washed with sterile distilled water twice.

Isolation and identification of antagonistic bacteria. The antagonistic bacteria were isolated from suppressive soils collected from Jungpyung, Eumsung, and Taejon where ginseng had been grown for several years without root rots. Soil samples (50 g) were suspended in 500 ml of sterile water and serial dilutions were made on soil extract agar medium. The plates were incubated at 29°C for 2 days and visible bacterial colonies were transferred to fresh LB agar medium. Potential antagonists against *B. cinerea* and *F. solani* were selected by dual culturing on potato dextrose agar (PDA) at 26°C for 6 days. Bacterial isolates which inhibit strongly mycelial growth of two pathogenic fungi were selected as potential antagonists. Identification of potential antagonistic bacteria was conducted according to Bergey's manual (1). Identification of selected isolates was further performed by Biolog GP Microplate System (11).

Effect of culture filtrates of bacterial antagonists on conidia germination of *B. cinerea* and *F. solani*. Four antagonistic bacteria (B-17, B-18, B-19 and B-19-17) were grown in LB broth at 29°C for 3 days. Culture filtrates were prepared by centrifugation of bacterial cultures at 8,000 rpm. Conidial suspensions of *B. cinerea* and *F. solani* (1×10^5 conidia/ml) were spread on water agar medium supplemented with bacterial culture filtrates by equal volume. The plates were incubated at 26°C and 17°C for *F. solani* and *B. cinerea*, respectively. Percentage of conidia germination was obtained by direct observation under microscope at least 100 conidia with 3 replicates. The experiments were repeated twice.

Colonization of bacterial antagonists on ginseng roots. The ability of cells of antagonistic bacteria to survive and multiply on the ginseng root was studied to determine if selected bacteria are effective colonizers of ginseng roots. Resistant strains to streptomycin (100 mg/ml) and rifampicin (50 mg/ml) were obtained from selected four antagonistic bacteria (B-17, B-18, B-19, and B-19-17) by UV-irradiation and selection on antibiotics amended media. These strains were tested for their antagonistic abilities by dual culturing with *B. cinerea* and *F. solani*. Their stabilities for antibiotics resistance were also tested through subculturing for 6 generations. These antibiotic resistant antagonistic bacteria were used for colonization and biological control tests. Surface sterilized 4-year-old ginseng roots were dipped into antagonistic bacterial suspension (10^7 cfu/ml) and placed into moistened clear aluminium boxes ($19\times14\times3.5$ cm). These boxes were incubated in a growth chamber at 26°C. To determine population density on ginseng roots, the ginseng root from each treatment was taken daily and completely homogenized in sterile distilled water. The population density was obtained by dilution plate assay on LB agar medium supplemented with streptomycin and rifamficin for 8 days with 1 day interval.

Biological control of ginseng root rots by antagonistic bacteria. Four-year-old ginseng roots were surface-disinfected with 3% sodium hypochlorites solution for 40 min and rinsed with sterile distilled water several times. These ginseng roots were dipped in antagonistic bacterial suspensions (B-17, B-18, B-19, and B-19-7; antibiotic resistant; 10^7 cfu/ml), procymidone (1 mg/ml), or distilled water. After drying at room temperature for 2 hr, the ginseng roots were inoculated by spraying conidial suspensions (1×10^5/ml) of *B. cinerea* and *F. solani*. Roots inoculated by *B. cinerea* and *F. solani* were incubated at 22 and 25°C, respectively. Disease severity (%) was measured 8 days after ino-

culation by estimating percentage of root rotting. Statistical computation was conducted by using the Statistical Analysis System (SAS Institute Inc., NC, USA).

Microfloral changes were monitored by the dilution plate techniques as described above. Population changes of *B. cinerea* and *F. solani* were monitored on 2% malt extract agar medium containing 5% oxgall and PCNB medium, respectively. Population changes of antagonistic bacterial were also measured on LB agar medium supplemented with streptomycin (100 m g/ml) and rifamficin (50 mg/ml). Population densities of fungal pathogens and antagonistic bacteria were measured for 8 days with 1 day interval.

Scanning electron microscopy. Four-year-old ginseng roots inoculated with *B. cinerea* and antagonistic bacterium B-19-17 simultaneously were fixed in 30% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) for 60 min. The specimens were washed three times for 10 min, postfixed in aqueous 2% OsO4 solution for 60 min, and rinsed 3 times with distilled water. Then the specimens were dehydrated through a series of ethanol-amyl acetate gradient. Critical point dried specimens were coated with gold and viewed in a scanning electron microscope (Hitachi S-570, Japan) in 10 Kv.

RESULTS

Isolation and identification of bacterial antagonists. Six suppressive soils to ginseng root rots were collected from Jungpyung, Eumsung, and Taejon. A total of 208 bacterial colonies were isolated from six soil samples and tested for their inhibitory effect on mycelial growth of *B. cinerea* and *F. solani*. Of 208 isolates tested, 4 (B-17, B-18, B-19, and B-19-17) have shown strong antagonistic activity (Fig. 1). These isolates also exhibited strong antagonistic activity against other plant pathogenic fungi including *Colletotrichum gloeosporioides, Pyricularia grisea, Rhizoctonia solani, Phomopsis sojae, P. longicola,* and *Phytophthora infestans* (data not shown).

Selected 4 bacterial isolates were gram positive and aerobes. They also formed endospores on 14 days after incubation on LB agar medium. According to the results of morphological and physiological characteristics and Biolog GP Microplate system, B-18, B-19, and B-19-17 were identified as *Bacillus amyloliquefaciens* with the similarity of 65.1, 54.9, and 57.0%, respectively. Isolates B-17 was identified as an unknown species of *Bacillus* (Table 1).

Effect of culture filtrates of bacterial antagonists on conidia germination of *B. cinerea* and *F. solani*. Conidia of *F. solani* in water control germinated 100% at 6 hr after incubation. However no conidia germination was observed at 6 hr incubation when culture filtrates of all 4 bacterial antagonists were added to the medium. When incubation was prolonged up to 16 hr, conidia germinated at reduced frequencies. Culture filtrate of B-19-17 was most potent, followed by those of B-19, B-18, and B-17, respectively (Table 2). Swelling and crushing of germ tubes were typical morphological abnormalities by culture filtrates in *F. solani* (Fig. 2). Conidia germination of *B. cinerea* was completely inhibited with all 4 culture filtrates up to 48 hr incubation, whereas most conidia germinated in control

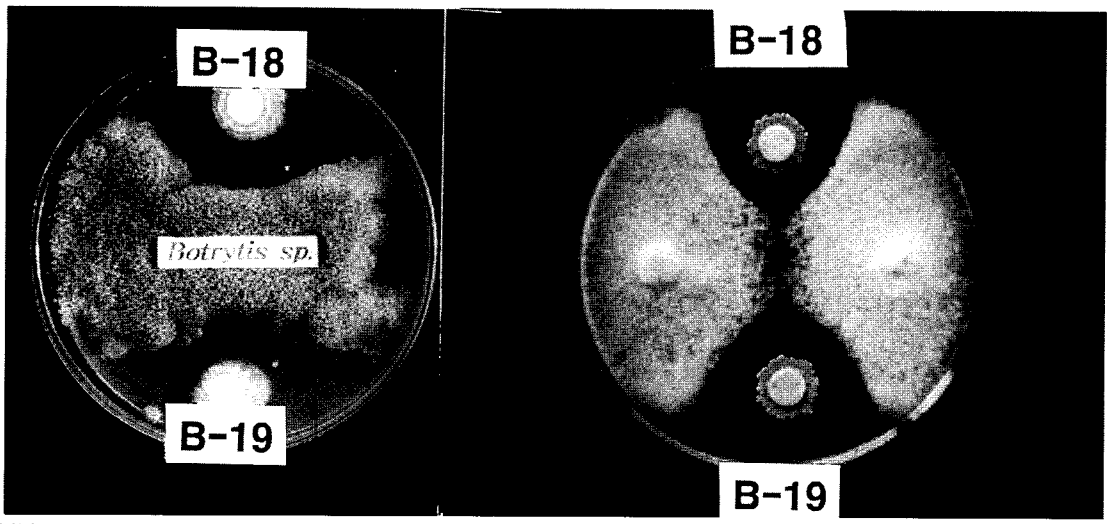

Fig. 1. Inhibitory effect of bacterial antagonists, B-18 and B-19, on the mycelial growth of *B. cinerea* (left) and *F. solani* (right) on PDA.

Table 1. Biochemical and physiological characteristics of antagonistic isolates compared with the description of *Bacillus amyloliquefaciens*

Tests	*Bacterial isolates*				*Bacillus amyloliquefaciens*
	B-17	B-18	B-19	B-19-17	
Gram reaction	+	+	+	+	+
Cell length (um)	1.5~4	2~3	2~3	2~3	1.8~3.0
Catalase	+	+	+	+	+
O/F test	−	−	−	−	−
Methyl red (MR) test	−	−	−	−	−
Voges-Proskauer (VP) test	+	+	+	+	+
Hydrolysis of Casein	+	+	+	+	+
Starch	+	+	+	+	+
Gelatin	−	+	+	+	+
Citrate	+	−	−	+	+
Nitrate reduction	+	+	+	+	+
Indole	−	−	−	−	−
H_2S	−	−	−	−	−
Growth in pH 6.8	+	+	+	+	+
5.7	+	+	+	+	+
Growth in NaCl 2%	+	+	+	+	+
5%	+	+	+	+	+
7%	+	+	+	+	+
10%	+	−	−	−	+
Growth at 10°C	−	−	−	−	−
30°C	+	+	+	+	+
40°C	+	+	+	+	+
55°C	−	−	−	−	−
Oxidase	−	−	−	−	−
Arginine dihydrolase	−	−	−	−	−
Lysine decarboxylase	−	−	−	−	−

Table 2. Effect of culture filtrates of bacterial antagonists on conidia germination of *B. cinerea* and *F. solani*

Isolates	Conidia germination (%+S.D.)			
	B. cinerea		*F. solani*	
	12 hr	48 hr	11 hr	16 hr
B-17	0	0	22.2±1.5	82.8±3.8
B-18	0	0	3.5±0.4	59.4±1.6
B-19	0	0	0.7±1.5	24.4±4.5
B-19-17	0	0	0.5±0.7	14.2±0.8
Water	91.4±6.2	91.4±6.2	100±0.0	100±0.0

Conidia germination was measured on water agar supplemented with culture filtrates of bacterial antagonists by equal volume.

by 12 hr (Table 2 and Fig. 3).

Root colonization of bacterial antagonists. To determine if selected bacteria are effective colonizers, the ability of antagonistic bacterial cells to survive and multiply in ginseng roots was evaluated. The population densities of 4 bacterial antagonists increased up to 3 days, and maintained without a large fluctuation up to 8 days on ginseng roots. The population densities of bacterial antagonists B-19-17 and B-19 were maintained at higher levels than those of B-17 and B-18 (Fig. 4).

Biological control of ginseng root rots by antagonistic bacteria. The control efficacy of root rots by antagonistic bacteria was evaluated. Treatments of all four antagonistic bacteria significantly reduced severity of root rots of ginseng by *B. cinerea* and *F. solani*. The control efficacies of root rots by antagonistic bacteria were comparable to those obtained by the treatment of a fungicide, procymidone. The antagonistic bacteria B-19 and B-19-17 were more effective than B-17 and B-18 to control both diseases (Table 3, Fig. 5 and Fig. 6).

The levels of population densities of *B. cinerea* and *F. solani* on ginseng roots increased up to 4 and 3 days, respectively, and then sustained the similar levels up to 8 days. Pre-treatments of ginseng roots with antagonistic bacteria significantly reduced the population levels of *B. cinerea* and *F. solani*. The procymidone treatment also reduced the population levels of two pathogens at the similar levels obtained by pre-treatments of antagonistic bacteria (Fig. 7 and Fig. 8).

Scanning electron microscopic observation showed that conidia of *B. cinerea* on ginseng did not germinate

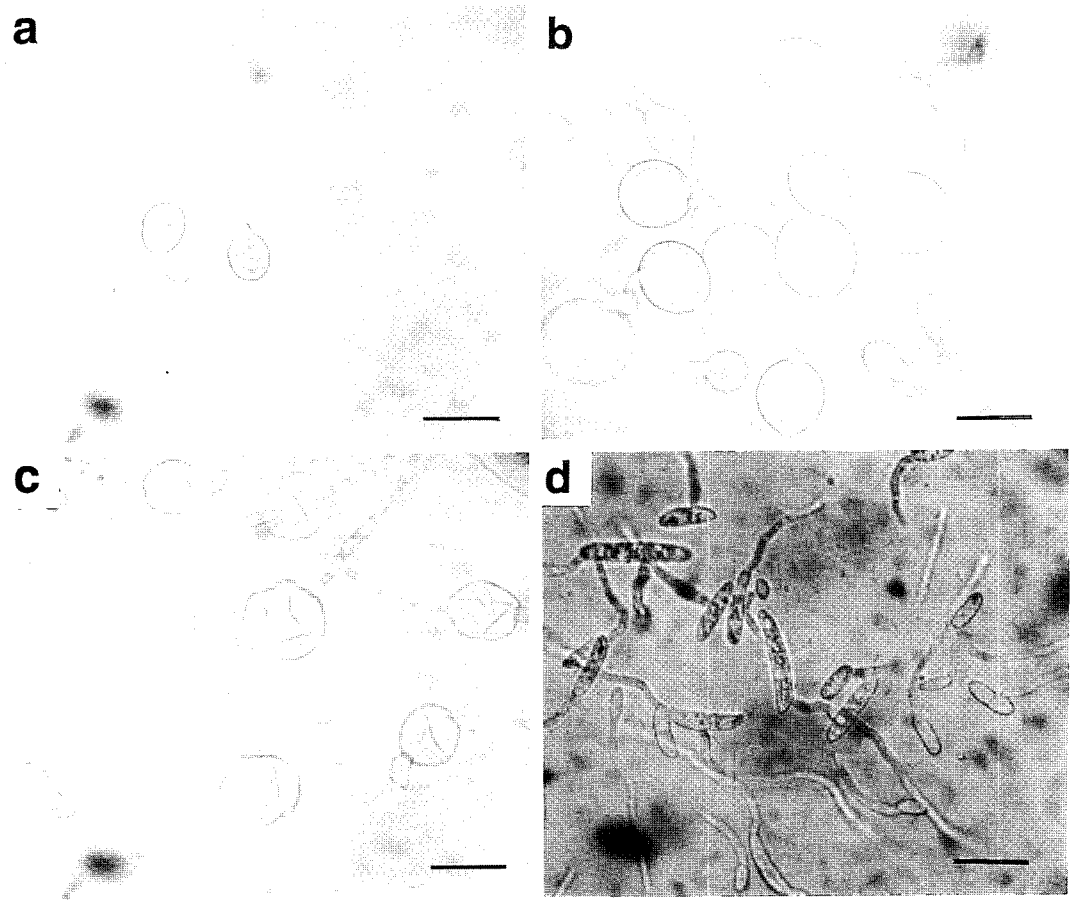

Fig. 2. Morphological abnormalities induced by culture filtrate of a bacterial antagonist B-19-17 on conidia germination of *F. solani*. a-c : germ tubes were swollen and crushed, d : normal germination. The scale bars represent 50 um.

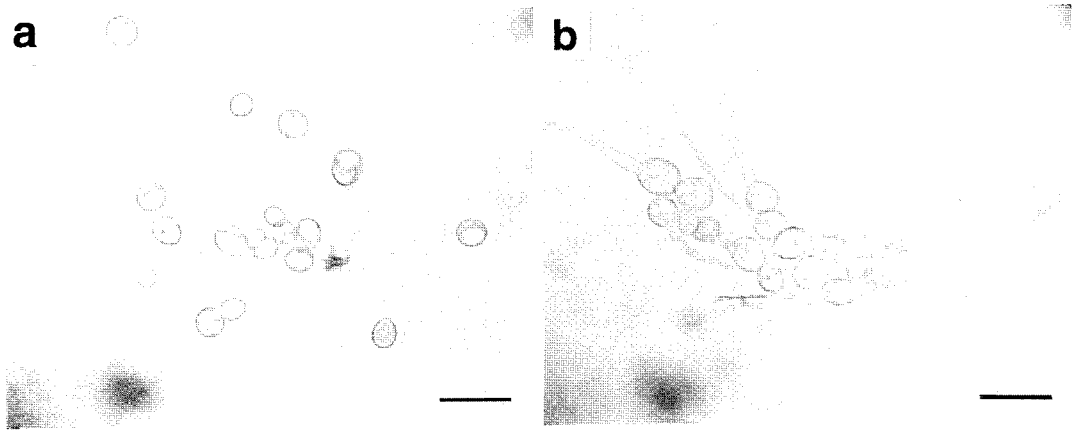

Fig. 3. Effect of culture filtrate of a bacterial antagonist B-19-17 on conidia germination of *B. cinerea*. a : conidia did not germinate until 48 hr after incubation and heavily vacuolated, b : normal germination at 12 hr of incubation. The scale bars represent 50 um.

and even distorted when coinoculated with the antagonistic bacterium B-19-17. These bacterial cells also covered around the conidia, hypha of *B. cinerea* and ginseng root (Fig. 9).

DISCUSSION

These experiments have shown that ginseng root rots by *F. solani* and *B. cinerea* during postharvest periods

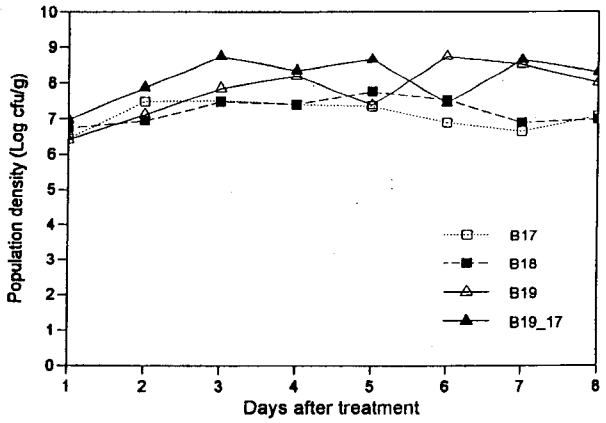

Fig. 4. Population changes of bacterial antagonists on ginseng roots. Ginseng roots were inoculated with 4 bacterial antagonists (10^7 cfu/ml) and incubated at ambient relative humidity.

Table 3. Effect of bacterial antagonists on the suppression of postharvest ginseng root rots by *B. cinerea* and *F. solani*

Treatment	Disease severity (%+S.D.)[x]	
	B. cinerea	*F. solani*
B-17	37.2±15.9b	40.4±14.1b
B-18	24.8±8.9b	35.3±22.2bc
B-19	20.8±2.2c	18.9±10.0cd
N-19-17	20.7±9.2c	26.9±12.3bcd
Procymidone	19.4±2.7c	9.4±3.0d
Water	100.0±0.0a	100±0.0a

[x] Disease severity (%) was measured at 8 days after inoculation. Means followed by the same letter are not significantly different at P=0.05 according to the Duncan's multiple range test.

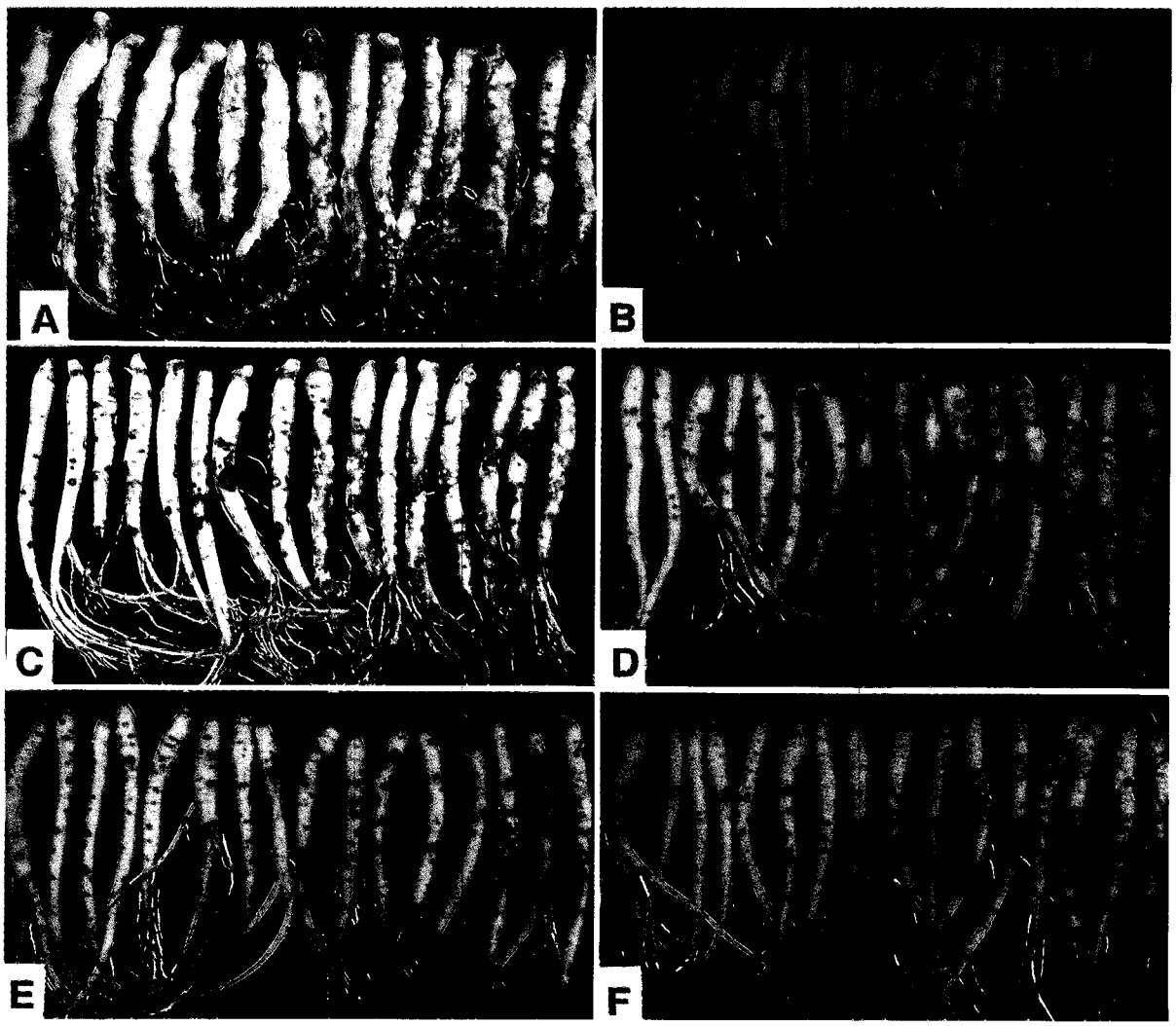

Fig. 5. Suppression of ginseng root rot caused by *F. solani*. Ginseng roots were treated with antagonistic bacteria or procymidone and subsequently inoculated with conidial suspension (10^5 conidia/ml) of *F. solani*. Disease severity was measured at 8 days after incubation. A : Water control, B : Procymidone, C : B-17, D : B-18, E : B-19, F : B-19-17.

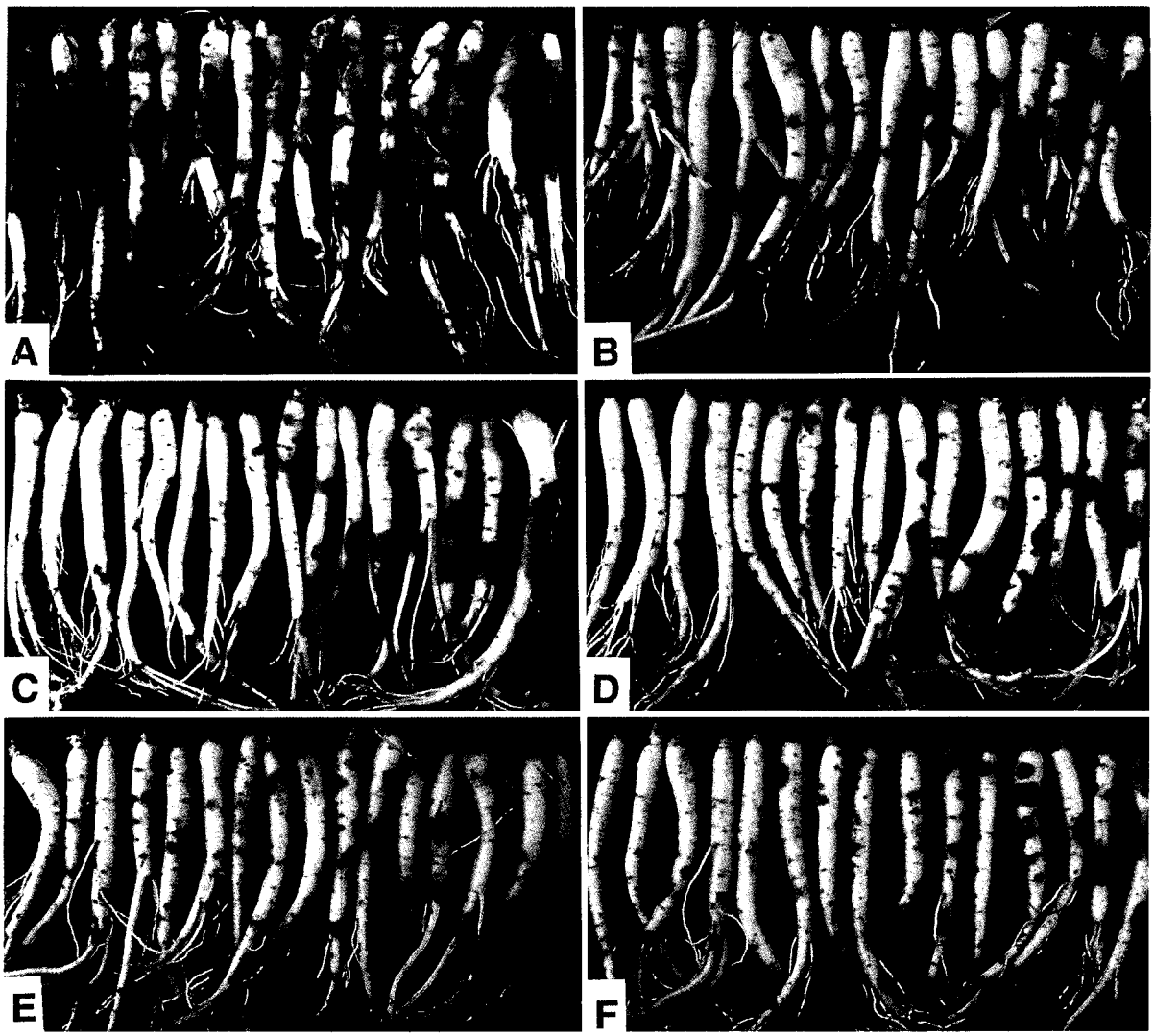

Fig. 6. Suppression of ginseng root rot caused by *B. cinerea*. Ginseng roots were treated with antagonistic bacteria or procymidone and subsequently inoculated with conidial suspension (10^5 conidia/ml) of *B. cinerea*. Disease severity was measured at 8 days after incubation. A : Water control, B : Procymidone, C : B-17, D : B-18, E : B-19, F : B-19-17.

could be reduced by the treatment of antagonistic bacteria isolated from suppressive soils. The bacterial antagonists B-18, B-19, and B-19-17 were identified as *B. amyloliquefaciens*, but B-17 was identified as unknown species of *Bacillus*. Highly amylolytic, spore-forming bacilli, *B. amyloliquefaciens*, was taxonomically separated from *B. subtilis* subspecies in 1968 (17).

The mechanism by which antagonistic bacteria inhibit the fungal pathogens of root rots have not been fully determined in these experiments. However, the culture filtrates of bacterial antagonists completely inhibited spore germination, and induced vacuolation of ungerminated conidia and mycelial swelling of *B. cinerea*. The culture filtrates also reduced significantly spore germination and induced hyphal tip swelling of *F. solani*. These data indicated that antibiotics are produced by these antagonistic bacteria. There is no report that *B. amyloliquefaciens* produces antifungal materials as yet. Although it has been reported that polyoxin D, a new class of peptide nucleoside, induced mycelial swelling of fungi by inhibiting chitin synthase activity (9), the clear mechanism of antifungal activity is not known in this experiment. Further research is remained to elucidate chemical properties of antifungal materials produced by *B. amyloliquefaciens.*

In the biological control of plant diseases, the ability of colonization by antagonists on the host surface is the key factor for the success. In this point, bacterial an-

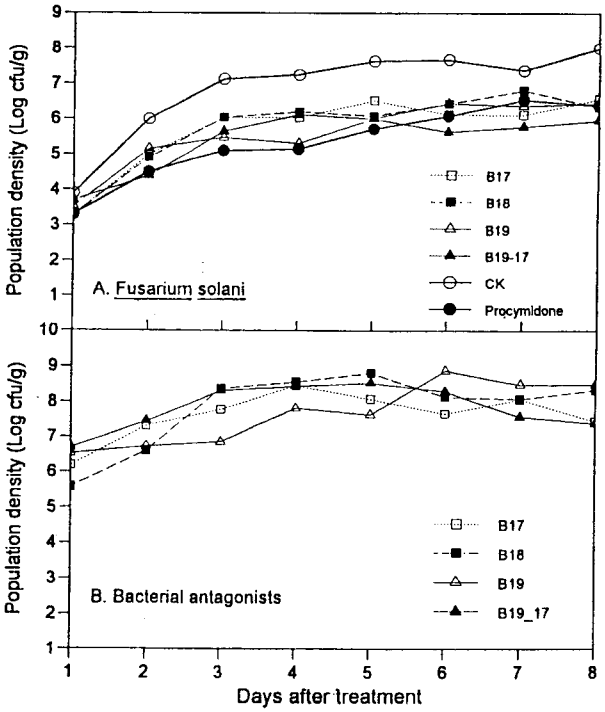

Fig. 7. Population changes of *F. solani* (A) and bacterial antagonists (B) on ginseng roots. Ginseng roots were treated with antagonistic bacteria (10^7 cfu/ml) or procymidone (1 mg/ml) and subsequently inoculated with conidial suspension (10^5 conidia/ml) of *F. solani.*

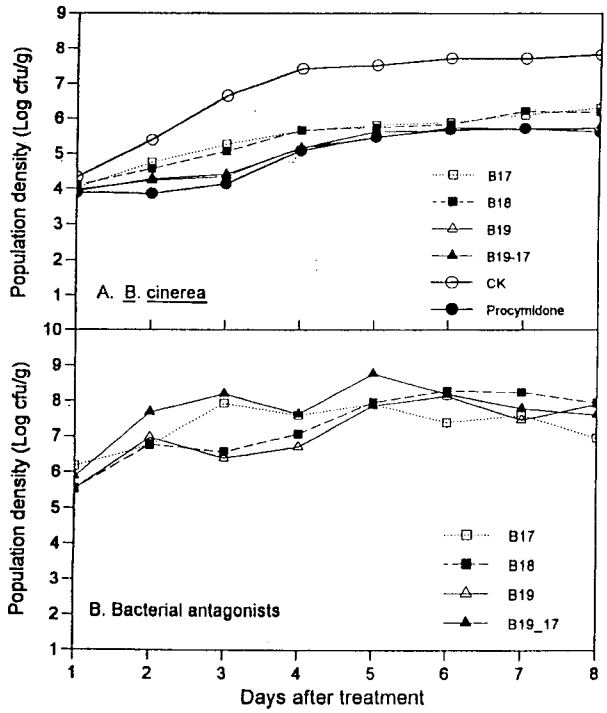

Fig. 8. Population changes of *B. cinerea* (A) and bacterial antagonists (B) on ginseng roots. Ginseng roots were treated with antagonistic bacteria (10^7 cfu/ml) or procymidone (1 mg/ml) and subsequently inoculated with conidial suspension (10^5 conidia/ml) of *B. cinerea.*

tagonists used in this experiment were effective colonizers on the surface of ginseng roots. The population densities of antagonists increased initially and maintained without a large fluctuation up to 8 days. Furthermore the population densities of bacterial antagonists were not affected even when the fungal pathogens were inoculated. In addition, colonization of bacterial antagonists on ginseng roots significantly reduced the population increase of the fungal pathogens. Indeed, ginseng roots colonized antagonistic bacteria have shown to have significantly fewer conidia and mycelium of fungal pathogens compared to untreated roots. These results also suggested that bacterial antagonists may produce antibiotics to retard conidia germination, which is absolutely required for disease initiation. Another explanation of these phenomena is that antagonistic bacteria were enable to exclude fungal pathogens by acquiring a greater portion of mutually required growth limiting resources. Nutrient and/or sites competitions between biological control agents and fungal pathogens have been determined to play important roles in biological control of plant diseases. In this case, effective attachment of bacterial cells to plant surface appears to be essential for biological control activity. Electron microscopic observation revealed that antagonistic bacteria adhered on the surfaces of ginseng roots as well as conidia of *B. cinerea* in these experiments. Taking together, antagonistic bacteria used in these experiments may use more than one mechanism to control ginseng root rots, although antibiotics seem to play a major role.

All experiments described here have been conducted at room temperature and the control efficacies of root rots by antagonistic bacteria were comparable to those obtained by the treatment of a fungicide, procymidone. Therefore the potential use of these antagonistic bacteria for biological control of ginseng root rots could be maximized at room temperature condition. Although positive results were obtained with antagonistic bacteria for the control of root rots in ginseng at postharvest stage, further research is required to increase and optimize the control efficacy in commercial packinghouse. Recently it has been demonstrated that antagonistic microorganisms are also capable of inducing resistance response in the host tissues (20). Furthermore, chitosan, a biodegradable food fiber, offers great potential as an antifungal preservative for fresh fruit and

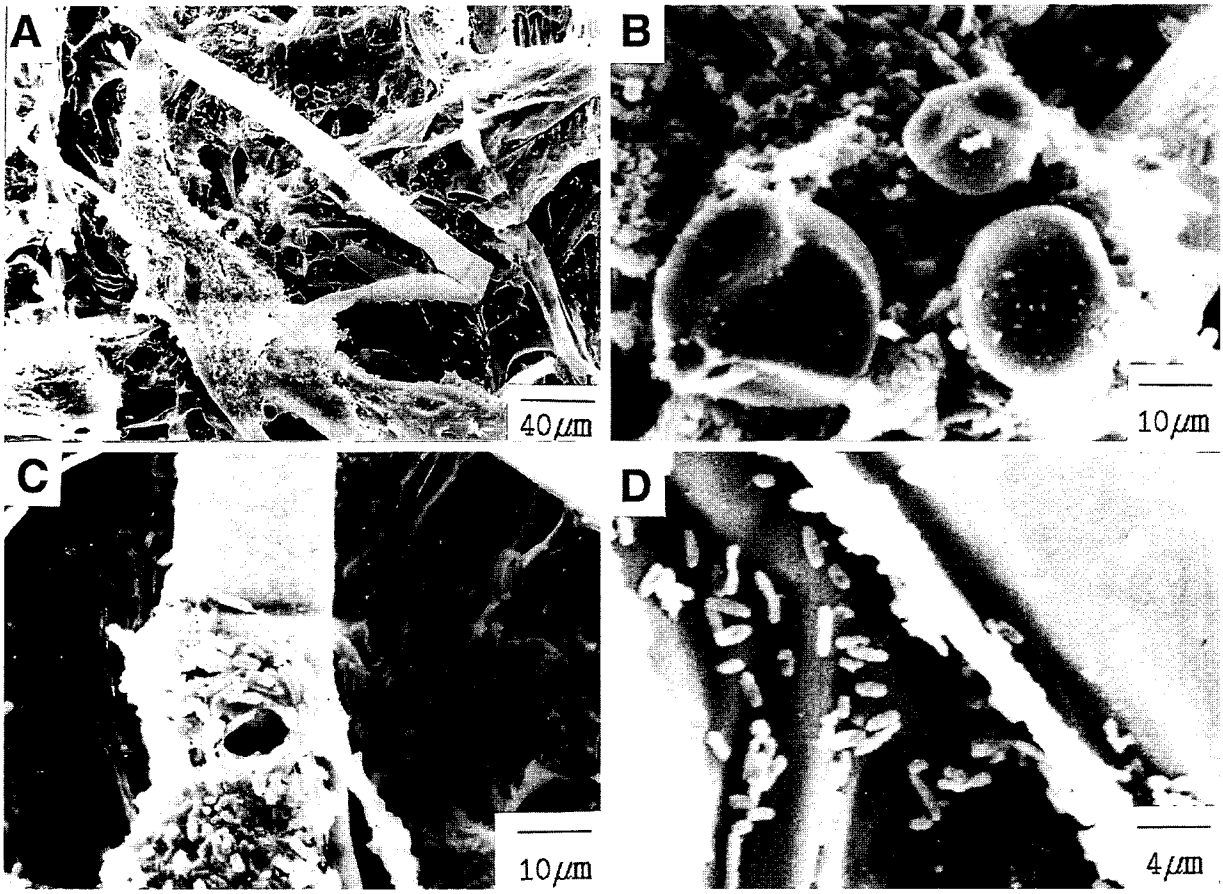

Fig. 9. Scanning electron micrographs of the antagonistic bacterium B-19-17 cells attached to hyphae (A : C shows the magnified region of A), conidia (B) of *B. cinerea,* and to ginseng root (D).

vegetables (8). These reports suggest that postharvest treatments of antagonists combining with other control practices for postharvest diseases perhaps hold the greatest promise for online practice.

요 약

인삼뿌리썩음병의 억제토양으로부터 썩음병을 일으키는 *Botrytis cinerea*와 *Fusarium solani*에 대한 길항성 세균을 선발하여 저장중의 인삼뿌리썩음병에 대한 생물학적 방제를 시도하였다. 분리된 208개 균주에서 길항력이 뛰어난 4개의 세균을 선발하였다. 이들은 *Bacillus amyloliquefaciens*(B-18, B-19, B-19-17)와 *Bacillus* sp. (B-17)로 동정되었다. 길항세균의 배양여액을 첨가한 물한천배지에서 두 병원균의 분생포자 발아는 현저히 억제되었다. *F. solani*의 분생포자는 대조구에서 6시간 후 100% 발아하였으나 처리구에서는 11시간 후부터 발아하기 시작하여 길항균의 종류에 따라 14~83%의 발아율을 나타내었다. 그러나 *B. cinerea*의 분생포자는 대조구에서 12시간 후 90% 이상 발아하였으나 처리구에서는 48시간 이후에도 전혀 발아하지 않았다. 실온에서 길항세균을(10^7 cfu/ml) 4년생 인삼에 처리하였을 때 *F. solani* 또는 *B. cinerea*에 의한 뿌리썩음병을 60~80% 방제할 수 있었다. 두 병원균의 밀도는 길항세균을 처리하지 않은 인삼표면에서 100~500배 높게 나타났으며 길항세균의 밀도는 병원균과의 동시접종 여부에 상관없이 일정 수준으로 유지되었다.

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