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Antagonistic Effect of Chitinolytic Bacteria on Soilborne Plant Pathogens

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토양전염성 식물병원균에 대한 Chitin 분해세균들의 길항효과

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ABSTRACT : One hundred and thirty bacterial isolates with high chitinolytic activity on chitin agar media were isolated and identified. Most of the isolates were *Aeromonas hydrophila* (110 isolates), and the others were *Serratia marcescens* (11 isolates), *Aeromonas caviae* (3 isolates), *Chromobacterium violaceum* strain C-61 (2 isolates), *Chromobacterium violaceum* strain C-72 (1 isolate) and unknown species (3 isolates). Among them, *C. violaceum* strain C-61 had highest chitinolytic activity and fungal growth inhibition on PDA. This bacterium also inhibited the growth of *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Phytophthora capsici* and *Pythium ultimum*, but it didn't inhibit the growth of *Fusarium oxysporum* and *Fusarium solani*. *C. violaceum* strain C-61 suppressed damping-off of eggplant caused by *R. solani*. Populations of the chitinolytic bacteria such as *Aeromonas hydrophila*, *Serratia marcescens*, *Aeromonas caviae*, *Chromobacterium violaceum* strain C-61 and *Chromobacterium violaceum* strain C-72 introduced into *R. solani*-infested soil were continuously decreased until 20 days after treatment, but their populations except *A. caviae* were not changed significantly and maintained over 5×10^4 CFU per g of soil thereafter.

Key words **: chitinolytic activity, *Chromobacterium violaceum*, damping-off of eggplant.**

In recent years, many studies have been made on the biological control of soilborne plant pathogens using antagonistic microorganisms as an alternative measure for the chemical control (7, 27). Antagonism has been shown to operate by antibiosis, competition, predation, and/or parasitism (10). Among them, the parasitism is involved in the lysis of cell wall of plant pathogenic fungi (4). Microorganisms capable of producing chitinolytic enzymes may degrade the fungal cell walls, of which a major component is chitin, and then inhibit the fungal growth (3, 21).

Arthrobacter sp., *Serratia liquefaciens* and *Hafnia alvei* protected carnation seedling from infection by *Fusarium oxysporum* f. sp. *dianthi* (16, 22, 23). *Serratia marcescens* suppressed *Sclerotium rolfsii* and *Rhizoctonia solani* in the greenhouse, but was not effective against *Pythium aphanidermatum* (17). *Stachybotrys elegans* (25), *Serratia marcescens* (8) and *Pseudomonas stutzeri* (14) had a strong antagonistic activity *in vitro* against *Rhizoctonia solani*, *Fusarium oxysporum* and *Fusarium solani*, respectively.

Microorganisms producing chitinolytic enzymes are known to be widespread in natural ecosystems and play an important role in the microbiological equilibrium in the soil (10). Therefore, this study was undertaken to isolate bacteria with high chitinolytic and antagonistic activity against soilborne plant pathogens.

MATERIALS AND METHODS

Isolation of chitinolytic microorganisms. Chitinolytic microorganisms were isolated from upland and

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paddy field soils in the eastern areas of Chonnam, Korea. Their populations were investigated by soil dilution plate method on the chitin agar plates (12) containing colloidal chitin (2 g/l) (6). Fungi, bacteria and actinomycetes producing clear zone (chitinolytic activity) on the chitin agar plates were counted after incubation at 28℃ for 3 days. One hundred and thirty chitinolytic bacteria with high chitinolytic activity were isolated and classified on the basis of colony types and physiological characteristics.

Identification and characteristics of the isolates. Physiological characteristics of isolated bacteria were investigated according to the Manual of Methods for General Bacteriology (9) and identified according to the Bergey's Manual of Systematic Bacteriology and of Determinative Bacteriology (11, 13). Morphological characteristics of the bacteria were investigated under an electron microscope. The bacterial isolates were preserved in 30% glycerol at −20 ℃ and in nutrient agar (NA) slant at 4℃.

Various enzymatic activities of the isolates were determined on proper media after 3-day incubation at 28℃. The media for enzyme activities are as follows; colloidal chitin agar for chitinolytic activity (22), β-glucan agar (Sigma, G 8902) for β-1,3-glucanolytic activity (19), skim milk agar for proteolytic activity, cellulolytic agar for cellulase activity of thermophiles, pectin medium for pectinase activity and Sierra medium for lipase activity described in Handbook of Microbiological Media (1).

***In vitro* fungal inhibition test.** Selected isolates were individually tested for their ability to inhibit mycelial growth of soilborne plant pathogens; *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Phytophthora capsici*, *Pythium ultimum*, *Fusarium oxysporum* and *Fusarium solani*. Four isolates were spotted on the edge of PDA plates and 0.5-cm-diameter PDA disks of the pathogen grown for 3 days at 28℃ were placed in the center of the plate. The plates were incubated at 28℃ for 4 or 5 days and the distances between the edges of the bacterial colony and fungal mycelium were measured.

Suppression of damping-off. Selected isolates were tested for their ability to control damping-off of the eggplant caused by *R. solani* in Wagner pot (15×6× 10 cm). *R. solani* strains were isolated from diseased eggplant and the inoculum was prepared as follows. The cultures of *R. solani* grown for 7 days on PDA were pulverized in a Waring blender, and then inoculated on the sterilized oatmeal-soil in 500 ml flasks. After incubation for 15 days at 28℃, the colonized oatmeal-soils were blended and sieved through a 0.25 mm sieve. The inocula were added to the sterilized soils at the rate of 1% (w/w) in Wagner pots.

Three-week-old seedlings were planted in two rows with 5 plants per row in Wagner pots filled with the infested soil. On the day of planting, 50 ml of bacterial suspensions (ca. 10^9 cells/ml) was drenched onto soil surface in each pot. Sterile distilled water was used as control. Damping-off incidence was measured every day after treatment. All experiments were conducted in a greenhouse with four replicates.

Population changes of introduced bacteria. The bacterial population in soils were measured on the chitin agar plates containing ampicillin, because all of the bacteria were resistant to ampicillin at 200 μg per ml.

RESULTS

Chitinolytic microorganisms. Many chitinolytic bacteria were present in soils, and estimated 1.8×10^4 living cells (CFU) per g of soil in upland and 8.5×10^3 CFU in paddy field soils. There were also many chitinolytic actinomycetes, although their populations were lower than those of bacteria. On the other hand, chitinolytic fungi were found in upland soils only with very low level (Table 1).

One hundred and thirty bacterial isolates with high chitinolytic activity on chitin agar media were selected and identified. Most of the bacteria were *Aeromonas hydrophila*, 110 isolates, and the other bacteria were *Serratia marcescens*, 11 isolates, *Aeromonas caviae*, 3 isolates, *Chromobacterium violaceum*

Table 1. Population density of chitinolytic organisms distributed in upland and paddy field soils

Chitinolytic organisms	CFU/g soil[a]	
	Upland field	Paddy field
Bacteria	1.8×10^4	8.5×10^3
Actinomycetes	5.6×10^3	3.7×10^2
Fungi	0.8×10^0	−

[a] Number of microorganisms forming the clear zone on chitin agar plates (22) after incubation at 28℃ for 3 days.

strain C-61, 2 isolates, *Chromobacterium violaceum* strain C-72, 1 isolate, and unidentified species, 3 isolates.

***In vitro* fungal inhibition and enzymatic activity.** The inhibition of *R. solani* growth on PDA plates was highest in *C. violaceum* strain C-61 and was followed by *C. violaceum* strain C-72. The other identified bacteria, *A. hydrophila*, *A. caviae* and *S. marcescens*, did not inhibit *R. solani* (Fig. 1).

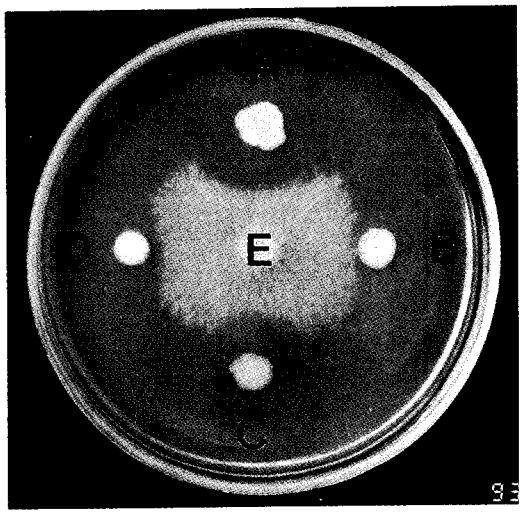

Fig. 1. Inhibition of mycelial growth of *R. solani* (E) by chitinolytic bacteria, A : *C. violaceum* C-61, B : *A. hydrophila*, C : *C. violaceum* C-71 and D : *S. marcescens*.

Two strains of *C. violaceum* inhibited the growth of *R. solani*, *S. sclerotiorum*, *Ph. capsici* and *Py. ultimum*, but *A. hydrophila*, *A. caviae* and *S. marcescens* did not inhibit, except for slight inhibition of *S. sclerotiorum* by *S. marcescens*. On the other hand, all the isolates did not inhibit the growth of *F. oxysporum* and *F. solani* (Table 2).

The enzymatic activities on agar plates containing respective substrate varied according to the isolates (Table 3). Chitinase was highest in *C. violaceum* strain C-61 and the following was strain C-72. Protease and lipase were highest in *A. hydrophila* and *S. marcescens*, respectively. DNAse was high produced only by *A. hydrophila*, *A. caviae* and *S. marcescens*. Pectinase was produced only by *S. marcescens*, but β-1,3-glucanase and cellulase were not produced in all of the isolates.

Suppression of Damping-off. The suppression of eggplant damping-off was highest in *C. violaceum* strain C-61 treatment and was followed by *C. violaceum* strain C-72 treatment. The disease incidence after 25 days was 18% in *C. violaceum* strain C-61 treatment, 40% in *C. violaceum* strain C-72 treatment,

Table 2. Inhibition of soilborne plant pathogens by chitinolytic bacteria on PDA plates

Bacterial strains	Inhibition zone (mm)[a]					
	R. solani	*S. sclerotiorum*	*Ph. capsici*	*Py. ultimum*	*F. oxysporum*	*F. solani*
A. hydrophila	<1	<1	<1	<1	<1	<1
S. marcescens	<1	1.8	<1	<1	<1	<1
A. caviae	<1	<1	<1	<1	<1	<1
C. violaceum C-61	7.5	10.8	3.7	7.3	<1	<1
C. violaceum C-72	5.3	<8.5	2.5	5.9	<1	<1

[a]The distance between the edges of bacterial colony and fungal mycelium.

Table 3. Enzymatic activity of chitinolytic bacteria on agar plates containing each substrate

Bacterial strains	Enzymatic activities[a]						
	Chi.[b]	Glu.	Prot.	Lip.	DNAse	Cell.	Pect.
A. hydrophila	++	−	++++	++	+++	−	−
S. marcescens	++	−	+++	+++	+++	−	++
A. caviae	++	−	++	++	+++	−	−
C. violaceum C-61	++++	−	++	++	−	−	−
C. violaceum C-72	+++	−	++	++	−	−	−

[a]The distance between the edges of bacterial colony and halo zone after incubation at 28℃ for 3 days, − : <1 mm, + : 1.1~2.0 mm, ++ : 2.1~3.0 mm, +++ : 3.1~4.0 mm, ++++ : 4.1~5.0 mm.

[b]Chi : chitinase, Glu : β-1,3-glucanase, Prot. : protease, Lip : lipase, Cell. : cellulase, Pect. : pectinase.

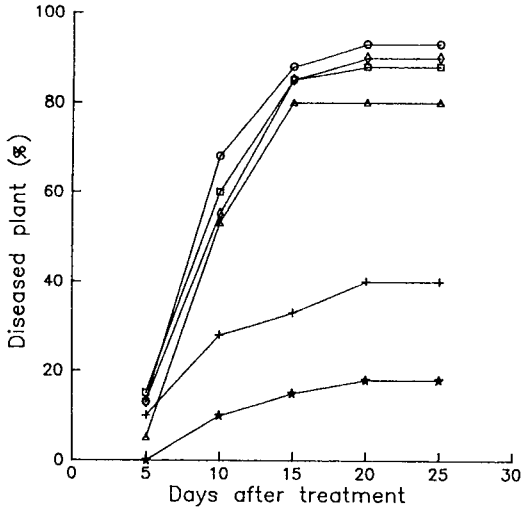

Fig. 2. Effect of chitinolytic bacteria on suppression of eggplant damping-off in the sterilized soil with inoculum of *R. solani*. *C. violaceum* C-61 (☆), *C. violaceum* C-72 (+), *A. hydrophila* (□), *A. caviae* (◇), *S. marcescens* (△) and untreated control (○).

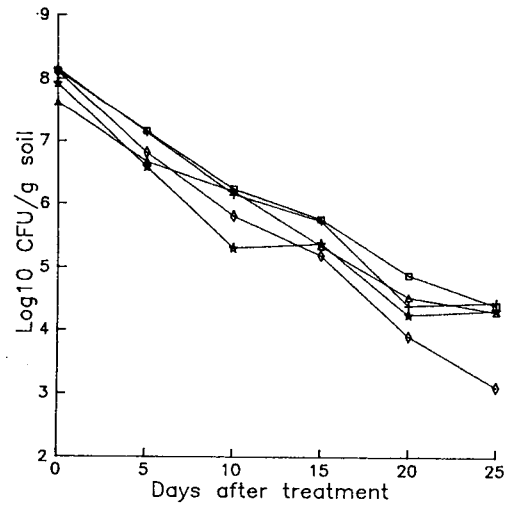

Fig. 3. Changes of population density of chitinolytic bacteria introduced in the sterilized soils with inoculum of *R. solani* and seedlings of eggplant: *C. violaceum* C-61 (☆), *C. violaceum* C-72 (+), *A. hydrophila* (□), *A. caviae* (◇), and *S. marcescens* (△).

and 80% in *S. marcescens* treatment. However, the disease incidence was not significantly reduced in *A. hydrophila* (88% incidence) and *A. caviae* (90% incidence) treatments, as compared with the nontreated control (93% incidence) (Fig. 2).

Population of the introduced isolates. All of the bacteria introduced into soil was continuously decreased until 20 days after treatment. In 20~25 days after treatment, the populations of *A. hydrophila*, *S. marcescens*, *C. violaceum* strain C-61 and strain C-72 were not significantly changed and maintained over 5×10^4 CFU per g of soil. However, the population of *A. caviae* was continuously decreased until 25 days after treatment (Fig. 3).

DISCUSSION

A large number of chitinolytic microorganisms, particularly bacteria and actinomycetes, were present in soils. They seem to play an important role in microbial equilibrium of the soils (10). The identified bacterial isolates, *Aeromonas hydrophila*, *Serratia marcescens*, *Aeromonas caviae* and *Chromobacterium violaceum*, have been already reported to be present in soils or water, and to produce chitinolytic enzymes (2, 5, 8, 11, 13, 15).

S. marcescens was known to suppress *Sclerotium rolfsii* and *Rhizoctonia solani* disease (17). However, *S. marcescens* obtained in this study did not inhibit mycelial growth of *R. solani*, *Ph. capsici*, *Py. ultimum*, *F. oxysporum*, and *F. solani*, although it slightly inhibited growth of *S. sclerotiorum*. Its ability to suppress damping-off caused by *R. solani* was not significantly different from the nontreated control. Also, *A. hydrophila* and *A. caviae* did not inhibit mycelial growth of all plant pathogens tested in our experiment and not suppress the eggplant damping-off. At present, studies on the fungal inhibition by *A. hydrophila* and *A. caviae* have not been reported yet, although the production and genes of chitinase by *A. hydrophila* have been studied (20, 28).

C. violaceum strain C-61 had very high antagonistic activity against all pathogens such as *R. solani*, *Ph. capsici*, *Py. ultimum* and *S. sclerotiorum*, except for *F. oxysporum* and *F. solani*. This isolate also significantly suppressed damping-off of eggplant caused by *R. solani*. Chitinolytic bacteria such as *Arthrobacter* sp., *Serratia liquefaciens* and *Hafnia alvei* (22, 23), *Serratia marcescens* (8, 17), *Pseudomonas stutzeri* (14) and *Stachybotrys elegans* (25) were reported to suppress soilborne plant pathogens. However, the antagonistic effects of *C. violaceum* against soilborne plant pathogens were only described in the book of Balows *et al.* (2) and its role in the rhizosphere

of plants is not clear yet, although *C. violaceum* strains isolated from rhizosphere soil of maize significantly increased the yield of dry matter of the plant when it is treated on maize seeds.

In chitinolytic microorganisms, chitinases and β-1,3-glucanases were reported to play an important role in the suppression of plant diseases (18, 25). Two strains of *C. violaceum* which had the strongest chitinolytic activity of all the bacterial isolates tested in this experiment showed high antagonistic effects on several soilborne plant pathogens. However, it is not clear yet whether such antagonistic effects were induced by chitinolytic enzymes or antibiotics, although there is a strong possibility of relationships between the enzyme activity and the antagonistic effect. On the other hand, two strains of *C. violaceum* could not degrade β-glucan on medium plates. This suggests that β-1,3-glucanase may not be involved in the disease suppression of the isolates.

The maintenance of the introduced bacteria in soils is an important factor for the effective control of root diseases. Moreover, their competitiveness with the rhizosphere microflora is one of the main conditions for a successful colonization (27). In our experiment, the population of the introduced *C. violaceum* was continuously decreased until 20 days after treatment, but maintained 5×10^4 CFU per g of soil 25 days after treatment. *C. violaceum* is mainly found in soil and water where it usually constitutes only a minor component of the total microflora, and is also occasionally an opportunistic pathogen for humans and animals (2). Therefore, further studies are needed to use *C. violaceum* as a biological control agent under the field conditions.

In the present study, we obtained various chitinolytic bacteria which differed in their ability to suppress soilborne plant pathogens. The characteristics of chitinase produced from the bacteria as well as the suppression mechanisms against plant diseases are needed in future studies.

요 약

Chitin 배지위에서 분해력이 더 큰 세균 130균주를 선발, 동정하였는데, 대부분의 균주(110 균주)는 *Aeromonas hydrophila*이었고, 그 나머지는 *Serratia marcescens*(11균주), *Aeromonas caviae*(3 균주), *Chromobacterium violaceum* strain C-61(2 균주), *Chromobacterium violaceum* strain C-72(1 균주) 등이었다. 이들 중 배지 위에서 chitin 분해력이 가장 컸던 *C. violaceum* strain C-61 균주는 병원균 생장억제력도 가장 컸다. 그것은 *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Phytophthora capsici*, *Pythium ultimum* 등을 억제하였지만 *Fusarium oxysporum* and *Fusarium solani*는 억제하지 못하였다. *R. solani*에 의한 가지 모잘록병도 다른 균주 보다 *C. violaceum* C-61에 의해서 더 억제되었다. *R. solani* 감염 토양에 처리된 균주의 밀도는 처리후 20일까지 계속 감소하였으나, 그 이후에는 *A. caviae*를 제외한 모든 균주가 크게 변화되지 않고 토양 1 g 당 5×10^4 CFU 이상의 밀도를 유지하였다.

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