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Antagonistic activity of *Streptomyces* species against *Fusarium solani* causing ginseng root rot

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인삼뿌리 썩음 병균 *Fusarium solani* 에 대한 *Streptomyces* species 의 길항작용

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ABSTRACT: Antagonistic effects of *Streptomyces* species aganinst *Fusarium solani* causing ginseng root rot were investigated in terms of chitinase activity and growth inhibition *in vitro*. Among 131 isolates of streptomycetes obtained from ginseng cultivating soil, 9 isolates producing large clear zone around the colony on a chitin agar medium were selected for further study. All 9 isolates produced chitinase in a range from 0.10 to 0.38 U lysing cells of *F. solani* and inhibited germination of the conidia. In the ten-fold concentrated culture filtrate of *S. alboniger* ST59 and *S. roseolilacinus* ST129, the number of conidia of *F. solani* was reduced to about 20% of original count within 14 days. When *S. alboniger* ST99 and *F. solani* were grown simultaneously in the mineral salt medium, chitinase activity increased with incubation period, whereas mycelial volume of *F. solani* decreased. In a chitin added mineral salt medium, chitinase activity increased during the first four days and maintained steady level until the 8th day, and increased thereafter. *S. alboniger* ST59 lysed mycelia, conidia and even chlamydospores of *F. solani*. It is probable that the antagonistic activity of this streptomycete against *F. solani* is the lysis of fungal cell wall by streptomycete producing chitinase affected by antifungal substances.

KEY WORDS□Chitinase, Streptomycetes, *Fusarium solani*.

The forms of antagonism generally recognized are antibiosis, competition, and predation or parasitism (Baker and Cook, 1974; Cook and Baker, 1983). Many bacteria, fungi, and actinomycetes have been shown to produce antibiotics. Actinomycetes, especially most streptomycetes produce antibiotics against fungal pathogens of plants (Broadbent *et al.*, 1971; Gottlieb and Siminoff, 1952; Old, 1965; Rangaswami and Ethiraj, 1962; Sneh and Henis, 1972). The parasitism by antagonistic microorganisms relies on lytic enzymes for the degradation of cell walls of pathogenic fungi. Chitinase and β-1,3-glucanase play an important role in the lysis of fungal cell walls, because most fungal cell walls contain chitin and β-1,3-glucan as major components (Bartnicki,

1973; Chet and Baker, 1980; Hadar *et al.*, 1978; Skujins *et al.*, 1965). The role of streptomycetes as producers of antibiotics and/or mycolytic enzymes, therefore, may be very important in the interaction between soil microorganisms despite their leisurely development and low competitiveness (Baker, 1968; Baker and Cook, 1974).

Ginseng root rot caused by *Fusarium solani* (Mart.) Appel & Wr. is one of main limiting factors in increasing yield. No practical control method for root rot is available, because ginseng is a perennial plant growing slowly in the same place for four or five years until harvest. Recently, biological control of soil-borne plant diseases has been attempted by the introduction of antagonistic microorganisms into soil or seedlings (Aldrich and Baker, 1970; Kawamoto and Lorbeer, 1976; Kommedahl and Windels, 1978). Chung *et al.* (1982) reported biological control of ginseng root rot by amendment with crab shell. The amendment reduced root rot due to mycolytic activity and/or antibiotic production by the increased streptomycetes (Chung and Kim, 1978).

In this study, we investigated the antagonistic mechanisms of *Streptomyces* spp. against *F. solani in vitro.*

MATERIALS AND METHODS

Microorganisms

Streptomyces spp. were isolated from ginseng cultivating soil using a colloidal chitin agar medium (Hsu and Lockwood, 1975). Nine isolates producing large clear-zone around the colony were selected among 131 *Streptomyces* and maintained on glycerol casein agar media at 4°C until use. Two isolates ST59 and ST129 were identified previously as *S. alboniger* and *S. roseolilacinus,* respectively (Chung *et al.*, 1982).

A root rot pathogen, *Fusarium solani* (Mart.) Appel Wr. was isolated from lesions of ginseng root rot and the pathogenicity was tested. The pathogen was maintained on potato dextrose agar media at 4 C. For observing lysis of *F. solani,* a living mycelium was prepared. *F. solani* was cultured in a 250 m*l* Erlenmeyer flask containing 100 m*l* potato dextrose broth on a rotary shaker (120 rpm) for 5 days at 30 °C. The mycelia were collected on a cheese cloth and washed with sterilized distilled water several times to remove nutrients, and then suspended in the sterilized mineral salt medium (1 mg dry wt/m*l*). The mycelial suspension was homogenized in a Waring blendor (31 BL 92) at low speed for 5 seconds. All the process of mycelial preparation was carried out under aseptic condition.

Culture Media

A colloidal chitin agar medium was used for the isolation of *Streptomyces* species. The chitin agar medium contained 1.5 g colloidal chitin and 20 g agar in one liter mineral salt medium. The colloidal chitin was prepared as follows; 5 g clean chitin (Kanto chemical Co., Tokyo, Japan) was moistened with acetone and dissolved in 100 m*l* cold concentrated hydrochloride. The chitin slurry was then filtered through glass wool pads and the colloidal chitin was washed with distilled water several times. Remaining acid in the colloidal chitin was neutralized with 1N NaOH.

The mineral salt medium described by Reynolds (1954) contained 0.7g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4.5H_2O$, 0.05 g $FeSO_4.7H_2O$, and 0.001 g $ZnSO_4$ in one liter distilled water. A glycerol casein agar medium consisted of 10 g glycerol, 0.3 g casein, 2.0 g KNO_3, 2.0 g NaCl, 2.0 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4.7H_2O$, 18 g agar, and one liter distilled water.

Chitinase activity

One m*l* spore suspension of each isolate of *Streptomyces* spp. was added to 50 m*l* aliquotes of a modified mineral salt medium with or without *F. solani* in an 100 m*l* Erlenmeyer flask. The modified mineral salt medium was prepared by adding 0.5 g yeast extract and 1.5 g colloidal chitin to one liter basic mineral salt medium described previously. Inoculated culture flasks were incubated for a certain period on a rotary shaker (120 rpm) at 30 °C. The cultures were filtered and the supernatant was used for measuring chitinase activity.

To determine chitinase activity, 1.0 m*l* colloidal chitin (4.0 mg/m*l*) and 2.0 m*l* 0.1M phos-

phate buffer (pH 7.0) were incubated with 2.0 m*l* of cell-free culture supernatant. After 60 min. incubation in a water bath at 50℃, 1.0 m*l* enzyme reaction mixture was pipetted into a screw cap tube containing 1.0 m*l* distilled water. The reaction was then stopped by placing the tube into a boiling water bath for 15 min. The tubes were cooled in tap water and centrifuged at 3,000 rpm for 20 min to remove residual chitin. The amount of n-acetylglucosamine (NAGA) present in 1.0 m*l* supernatant was determined according to the method described by Reissig *et al.*(1955). A unit of chitinase activity (CHU) was defined as the amount of enzyme required to release 1 μmole of NAGA under the described condition.

Antagonistic activity

Antagonistic activity of *Streptomyces spp.* against *F. solani* was determined by measuring conidial germination, germ tube length, and mycelial lysis.

Conidial germination was determined by placing a drop of conidial suspension of *F. solani* on the back of culture discs or in culture filtrate of *Streptomyces* spp. grown on or in a glycerol casein medium at 30℃. Mycelial lysis of *F. solani* in the presence of *Streptomyces* spp. was rated on a scale from 0 to 4 after 7 days incubation by observation of ten microscopic fields of each treatment. The scale 0-4 represented; 0: no lysis, 1: 1-10 %, 2: 11-50 %, 3: 51-90 %, and 4: 91-100 % of mycelia lysed. To determine mycelial lysis in terms of volume, 30 m*l* mycelial suspension of *F. solani* and 10 m*l* spore suspension of *S. alboniger* ST59 were mixed in an 100 m*l* Erlenmeyer flask and cultured on a rotary shaker with 120 rpm at 30 ℃. The volume of the mixture was measured with a 2.5 m*l* capacity hypodermic syringe after centrifugation at 3,000 g for 20 min. Mycelial volume of *S. alboniger* ST59 was negligible comparing to that of *F. solani.*

RESULTS

Inhibition of conidial germination

Five isolates of *Streptomyces* spp. ST59, ST 118, ST122, ST128, and ST129 inhibited conidia germination of *F. solani* more than 90% after 24 hrs incubation in 2 week-old culture filtrate (Table 1, and 2). The growth of germ tube was also inhibited by *S. alboniger* ST59 and *S.*

Table 1. *Antagonistic activity of nine Streptomyces species isolated from ginseng cultivating soils against Fusarium solani.*

Streptomyces isolates	Chitinase activity (CHU)[a]	Conidial germination(%)[b] Hours after incubation 4	24	Lysis[c] rating
ST 7	0.29	45.3	90.0 b[d]	1
ST 59	0.20	6.7	9.5 d	4
ST 104	0.15	1.8	94.2 ad	1
ST 118	0.38	0	10.0 d	3
ST 120	0.29	83.5	100.0 a	3
ST 121	0.38	12.0	60.0 c	1
ST 122	0.34	2.2	8.0 d	2
ST 128	0.35	0	0.5 d	2
ST 129	0.10	0.8	4.7 d	1
Control	0	51.8	98.2 ab	0

[a] Chitinase activity (CHU) was determined as micromoles of N-acetylglucosamine per hour in 1 m*l* of culture filtrate.

[b] Conidia were treated with 2-week-old culture filtrate of each *Streptomyces* species.

[c] Lysis of mycelia was rated 7 days after incubation in culture filtrate of *Streptomyces* according to the following scale; 0: No lysis, 1: 1-10%, 2: 11-50%, 3: 51-90%, 4: 91-100% of mycelia lysed.

[d] In each column, values followed by the same letter do not differ significantly (p = 0.05) according to Duncan's multiple range test.

Table 2. *Inhibition of conidial germination and growth of germ tubes of Fusarium solani on the agar disc preinoculated with Streptomyces species.*

Streptomyces isolates	Conidial germination[a] (%)	Length of germ tube[b] (μm)
S. alboniger ST59	8.4	104 ± 64
S. roseolilacinus ST129	2.4	< 60
Control	90.6	520 ± 144

[a] Conidial germination and germ tube length were measured 12 hours after incubation on the glycerol casein agar disc preinoculated with *Streptomyces* species for 5 days. Average percent germination based on the examination of 5 fields on each of 3 plates with 30-50 condia per field.

[b] Germ-tube length is a mean of 10 replicates.

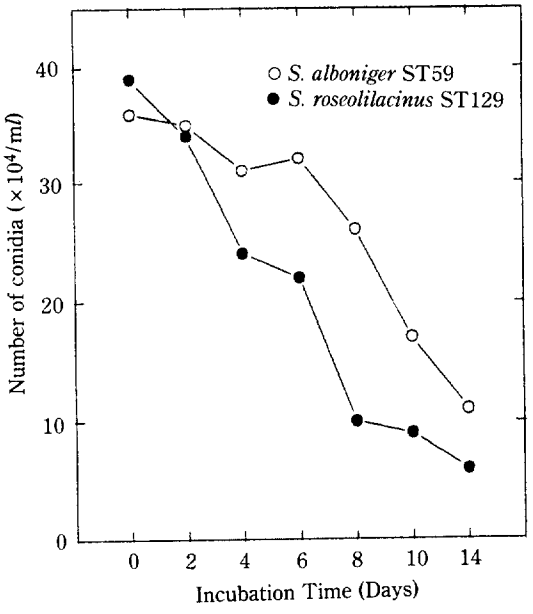

Fig. 1. *Decrease in the conidial number of Fusarium solani in culture filtrate of Streptomyces alboniger ST59 (○) and Streptomyces roseolilacinus ST129 (●).*
Conidia germinated and grew actively to form mycelial mat in culture media without *Streptomyces* inoculation.

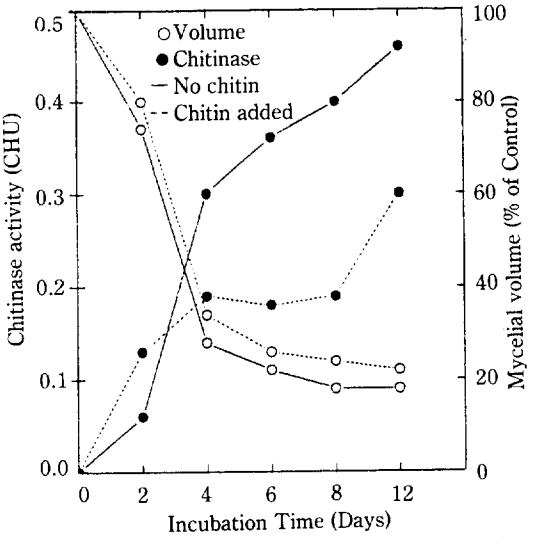

Fig. 2. *Chitinase production by Streptomyces alboniger ST59 inducing lysis of the mycelia of Fusarium solani when both organisms were grown simultaneously in mineral salt media.*
One hundred and twenty mg colloidal chitin was added to 40 m*l* of mixed culture in the medium.

roseolilacinus ST129 (Table 2).

The length of *Fusarium* conidia incubated on the culture disc of *S. alboniger* ST59 and *S. roseolilacinus* ST129 was 104 μm and less than 60 μm, respectively, comparing to that of control, 520 μm. In ten-fold concentrated culture filtrate of two *Streptomyces* spp., the number of conidia was decreased to about 20 % of original count within 14 days (Fig. 1). On the contrary in control without culture filtrate, it was difficult to count condia from two days after incubation due to active mycelial growth of *F. solani*.

Chitinase production

All nine *Streptomyces* spp. produced chitinase in a range from 0.10 to 0.38 inducing lysis of *F. solani* (Table 1). When *S. alboniger* ST59 and *F. solani* were mixed and cultured simultanlously, chitinase production was increased rapidly from the second day to the fourth day after treatment. The activity was then reduced. In the mixed culture added with colloidal chitin, the chitinase activity was higher than that in the control without chitin for the first two days. However, from the fourth day the activity was much less than that of control and kept a steady state until the 8th day and increased again, thereafter.

Mycelial lysis

Mycelial lysis observed under the microscope ranged from 1 to 4 on a rating scale. *Streptomyces* ST59, ST118, and ST120 showed strong lytic activity against *F. solani* (Table 1).

Among tested *Streptomyces* isolates, *S. alboniger* ST59 showed strongest lytic activity. This isolate lysed the mycelia, macrocondia and even chlamydospores of *F. solani* when both microorganisms were grown simultaneously for six days in the mineral salt medium (Fig. 3 A and B). Mass reduction of *F. solani* in the mixed culture with ST59 occurred from the second day to the fourth day after treatment and followed by gradual decrease. The reduction of mycelial volume in the chitin added solution was less than that in the control (Fig. 2).

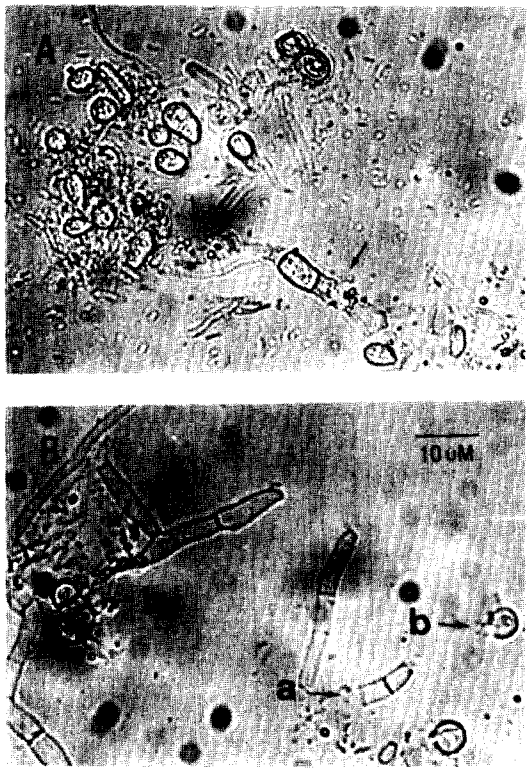

Fig. 3. *Lysis of Fusarium solani by Streptomyces alboniger ST59.*
Both microorganisms were seeded simultaneously in the mineral salt medium and incubated for six days at 30°C. A. Lysis of mycelia, B. Lysis of a macroconidium(a) and chlamydospores(b).

DISCUSSION

The production of chitinase and/or antibiotics by nine *Streptomyces* spp. might induce antagonistic activity against *F. solani* (Table 1). No correlation between chitinase activity, inhibition of conidial germination and the rate of mycelial lysis, respectively, indicates that these activities of streptomycetes are not always involved in the lysis of *F. solani*. This is partly in disagreement with the findings by Lloyd *et al.* (1965), who suggested the lysis of mycelia be correlated with the size of inhibition zone of *Glomerella cingulata* and not to be associated with chitinase activity by streptomycetes. It appears that other lytic enzyme, β-1,3-glucanase in addition to chitinase may be involved in the lysis of *F. solani* (Horikoshi and Lida, 1959; Potgieter and Alexander, 1966; Skujins *et al.*, 1965). The lysis could be an autolysis induced by antibiotics or heterolysis by chitinase (Lloyd *et al.*, 1965; Lloyd and Lockwood, 1966).

The strong inhibition of conidial germination of *F. solani* in concentrated culture filtrate of *S. alboniger* ST59 and *S. roseolilacinus* ST129 indicates the production of antibiotics (Table 2). Conceivably, the reduction of conidial numbers (Fig. 1) might result from autolysis induced by antibiotics not from streptomycetes chitinase, because chitinase, an inducible enzyme, could not be produced in media without the substrate (Reynolds, 1954; Skujins *et al.*, 1965). It is well known that most streptomycetes produce antibiotics and also lyse chitin, the constituent of most fungal cell wall (Potgieter and Alexander, 1966; Rangaswami and Ethiraj, 1962).

The results of the present study demonstrated that chitinase activity increased in contrast with the decrease of mycelial volume of *F. solani* with incubation time (Fig. 2). The mass reduction might be due to the lysis caused by chitinase affected by antibiotics. Addition of colloidal chitin to the culture mixture kept chitinase activity from being increased during the incubation period. Morrissey *et al.* (1976) suggested that the rate of chitinase production was limited by the rate of uptake of the hydrolytic products and when utilizable chitin was added to the culture, catabolite repression occurred. Possibly, in this mixed culture of *F. solani* and *S. alboniger* ST59, the catabolite repression of chitinase synthesis might occur.

Lysis of conidia and even chlamydospore of *F. solani*, a major form in soil and more resistant to enzymic degradation, suggests that the suppression of the pathogen by streptomycetes producing antibiotics and chitinase occur in soil.

적 요

Chitin 분해 활성이 높은 *Streptomyces* 균주들을 인삼재배 토양에서 분리하고, 그 균주들이 인삼뿌리 썩음 병균인 *Fusarium solani*에 미치는 길항효과와 그 작용을 조사하였다. 분리된 *Streptomyces*중 몇 균주는 *F. solani*의 포자발아와 발아관의 생장을 억제하였고 균사를 분해하였으며, 동시에 세포벽 분해효소로 알려진 Chitinase를 생산하였다. 그중에서도 *S. alboniger* ST 59와 *S. roseolilacinus* ST 129는 길항효과가 아주 좋았는데, 병원균의 분생포자를 두 *Streptomyces*의 농축배양여액에 14일간 처리하였을 때 포자숫자가 처음 접종 정도의 20%로 줄어들었다. 특히 *S. alboniger* ST 59는 병균인 *F. solani*의 분생포자, 균사 뿐만 아니라 분해되기 어려운 후막포자까지도 분해하였다. 이것으로 미루어 보건데, *F. solani*의 이 *Streptomycetes*에 의한 억제작용은 항생물질에 영향을 받은 병균이 Chitinase에 의해 분해된 것으로 생각된다.

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