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Production of an anti-fungal substance for biological control of *Phytophthora capsici* causing phytophthora blight in red-peppers by *Streptomyces halstedii*

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Abstract

The culture broth of *Streptomyces halstedii* AJ-7 suppressed the growth of *Phytophthora capsici*, which causes phytophthora blight in red-peppers, with less than 1% survival of the pathogen after 12 h of treatment. The low molecular fraction ($\leqslant$10 kDa) of the culture broth retained anti-fungal activity against *P. capsici* after being held at 100 °C for 6 h.

Introduction

Red-peppers (*Capsicum annum* L.) are widely cultivated around the world. Phytophthora blight, which is a serious problem in their cultivation, is caused by *Phytophthora capsici* (Akihiri *et al.* 1992). The pathogen is usually controlled with chemicals but their excessive use has led to environmental pollution. Such chemicals can also kill useful soil insects and beneficial microorganisms in the rhizosphere; they may also enter the food chain (Bartlett *et al.* 2002). Moreover, their efficiency is decreased due to the evolution of resistant pathogens (Rosenberger & Meyer 1981). Over the last 25–30 years, alternative control methods, including the use of biological control microorganisms, have been attempted (Mandeel & Baker 1991, Larkin *et al.* 1993).

Actinomycetes have been investigated as broad-spectrum biological control agents for fungal plant pathogens (Pedziwilk 1995, El-Tarabily *et al.* 2000). In the present study, the potential of *Streptomyces halstedii* AJ-7 was evaluated to control *Phytophtora capsici* as a soil-borne pathogen of red-peppers.

Materials and methods

Bacterial strain and culture conditions

Streptomyces halstedii AJ-7 was isolated from soil (unpublished data). It was cultured in a modified Bennet medium (10 g glucose, 2 g peptone, 1 g beef extract, 1 g yeast extract, 10 g colloidal chitin, 0.5 g $MgSO_4$, 0.5 g K_2HPO_4, 1 l water, pH 7.2) at 28 °C for 5 d. The culture was centrifuged ($1000 \times g$ at 4 °C for 20 min) and the supernatant was used for anti-fungal activity.

The phytopathogenic fungi, *Colletotrichum gloeosporioides* KCTC6169 and *Fusarium oxysporium* KCTC16341, were obtained from the Korean Collection for Type Cultures, Daejeon, Korea. *Alternaria alternata*, *Botrytis cinerea*, *Phytophthora capsici*, *Pythium ultimum*, *Rhizopus stolonifer*, and *Stemphylium lycopersici* were from the Laboratory of Phytopathology, Kyungpook National University, Daegu, Korea. Fungi were incubated at 28 °C on a potato/dextrose agar (PDA; Difco, USA).

To obtain spores of *Phytophthora capsici*, mycelia were grown on a potato/dextrose (PD) liquid medium at 28 °C for 7 d and the spores

were collected by filtration and centrifugation ($1000 \times g$ at 4 °C for 10 min). The spore density was then adjusted to 10^9 spores per ml with sterile distilled water.

Anti-fungal activity test

Anti-fungal activity was estimated by a growth inhibition assay. The fungal spores (10^6 spores per ml) were spread on a PDA plate and a paper disk containing a cell-free culture broth of *S. halstedii* AJ-7 (40 μl) was then placed in the center of the plate. After 5 d at 28 °C, the growth of the fungus was measured. The degree of inhibition was divided by the range of clear zone. The anti-fungal activity by the culture broth was classified as no inhibition (−; $\leqslant$2 mm), weak inhibition (±; 2–3 mm), moderate inhibition (+; 3–10 mm), strong inhibition (++; 10–20 mm), or very strong inhibition (+++; $\geqslant$20 mm).

Fractionation of S. halstedii *AJ-7 culture broth*

The cell-free culture broth of *S. halstedii* AJ-7 was fractionated into a high molecular weight component (high molecular fraction; $\geqslant$10 kDa) and a low molecular component (low molecular fraction; $\leqslant$10 kDa) by ultrafiltration through an YM10 membrane (Ø43 mm; Amicon Co., MA).

Biological control of phytophthora blight on red-pepper plug seedlings

Red-pepper seeds were soaked in the culture broth, the low molecular fraction, and the high molecular fraction of *S. halstedii* AJ-7 for 2 h. The control seeds were treated with distilled water. After being sown, red-pepper plug seedlings were grown in wet potting soil inoculated with spores of *Phytophtora capsici* (10^6 spores per g soil), which causes phytophthora blight in red-peppers. Red-pepper plug seedlings were grown as reported previously (Joo *et al.* 2004). During 30 d plant culture, the suppression of phytophthora blight by the culture broth of *S. halstedii* AJ-7 was measured in 105 seedlings.

Results and discussion

Anti-fungal activity of S. halstedii *AJ-7*

The low molecular fraction of *S. halstedii* AJ-7 suppressed the growth of various fungal phytopathogens: soil-borne pathogens (*Phytophtora capsici* and *Pythium ultimum*), foliar plant pathogens (*A. alternata, B. cinerea, C. gloeosporioides* and *Stemphylium lycopersici*), and post-harvest storage pathogens (*F. oxysporium* and *R. stolonifer*) (Table 1).

Table 1. Anti-fungal activity of *Streptomyces halstedii* AJ-7 against red-pepper pathogens.

Red-pepper pathogens (disease)	Degree of inhibition[a]	
	Low molecular fraction[b]	High molecular fraction[b]
Alternaria alternata (black mold)	++	++
Botrytis cinerea (gray mold)	++	–
Colletotrichum gloeosporioides (anthracnose)	++	++
Fusarium oxysporium (fruit rot)	++	+++
Phytophthora capsici (phytophthora blight)	+++	++
Pythium ultimum (damping-off)	+	±
Rhizopus stolonifer (rhizopus fruit rot)	++	–
Stemphylium lycopersici (leaf spot)	++	+

S. halstedii AJ-7 was cultured in a modified Bennet medium at 28 °C for 5 d. A cell-free culture broth was used for anti-fungal activity.
[a]The degree of inhibition was divided by the range of clear zone. Anti-fungal activity by the cell-free culture broth of *S. halstedii* AJ-7 was classified as no inhibition (−; $\leqslant$2 mm), weak inhibition (±; 2–3 mm), moderate inhibition (+; 3–10 mm), strong inhibition (++; 10–20 mm), or very strong inhibition (+++; $\geqslant$20 mm).
[b]The culture broth was fractionated into a high molecular weight component ($\geqslant$10 kDa) and a low molecular component ($\leqslant$10 kDa) by ultrafiltration through a 10 kDa membrane.

Table 2. Anti-fungal effect of *Streptomyces halstedii* AJ-7 culture broth against *Phytophthora capsici*.

Treated with culture broth (h)	Survival rate of *Phytophthora capsici* (%)	
	Culture broth	Heated culture broth[a]
0	100	100
2	36 ± 2	56 ± 3
4	24 ± 5	43 ± 4
6	16 ± 4	30 ± 1
8	7 ± 1	26 ± 5
10	4 ± 2	18 ± 3
12	1 ± 3	13 ± 4
14	0 ± 1	10 ± 2
24	0 ± 1	0 ± 1

[a]A cell-free culture broth was treated at 80 °C for 30 min. The remaining anti-fungal activity was assayed by the germination inhibitory ratio. Spores of *Phytophthora capsici* (10^6 spores per ml) were inoculated in a potato/dextrose broth and 20% (v/v) of the culture broth was added. The resultant solution was incubated at 28 °C. Samples were taken at various intervals and spread on a potato/dextrose agar plate. All assays were performed in triplicate.

Growth suppression of Phytophtora capsici *by culture broth of* S. halstedii *AJ-7*

The culture broth of *S. halstedii* AJ-7 suppressed the growth of *Phytophthora capsici* with less than 1% survival of the pathogen after 12 h of treatment (Table 2). The culture broth treated at 80 °C for 30 min also retained anti-fungal activity, suggesting that a thermostable anti-fungal material was present in the culture broth.

Table 3. Thermostability of low molecular fraction of *Streptomyces halstedii* AJ-7.

Heat treated time (h)	Relative activity (%)		
	80 °C	90 °C	100 °C
0	100	100	100
2	100	100	96
4	100	99	86
6	100	95	53
8	100	80	30
10	98	60	24
12	95	56	9

A culture broth of *S. halstedii* AJ-7 was fractionated into a low molecular fraction as described in Table 1. The low molecular fraction was treated at 80, 90, and 100 °C for the indicated times. The remaining anti-fungal activity was estimated by measuring the growth inhibition of the fungus. The fungal spores (10^6 spores per ml) were spread on a potato/dextrose agar plate and a paper disk containing the low molecular fraction (40 μl) was then placed in the center of the plate. After 5 d incubation at 28 °C, the growth of the fungus was measured. All assays were performed in triplicate.

Thermostability of low molecular fraction of S. halstedii *AJ-7*

After heat treatment at 80 °C for 30 min, the high molecular fraction *of S. halstedii* AJ-7 lost anti-fungal activity (data not shown) but the low molecular fraction retained anti-fungal activity against *Phytophthora capsici* (Table 3). The half-life of the low molecular fraction was 6.3 h at 100 °C. From the above results, it was concluded that a thermostable anti-fungal material was present in the low molecular fraction.

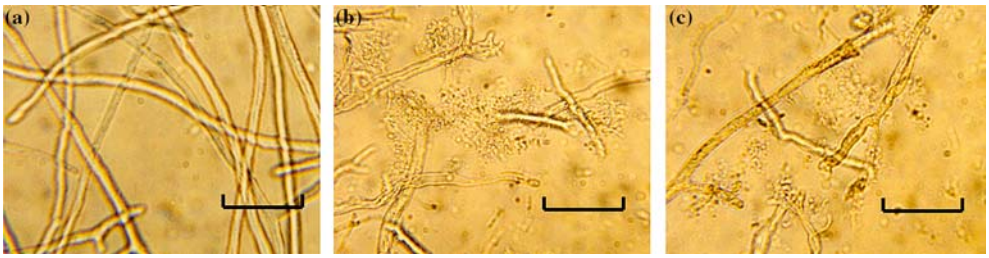

Fig. 1. Abnormal hyphal morphology of *Phytophthora capsici* caused by a culture broth of *Streptomyces halstedii* AJ-7. Spores of *P. capsici* (10^6 spores per ml) were pre-inoculated for 24 h in a potato/dextrose broth. Twenty % (v/v) of low molecular fractionation or high molecular fractionation of *S. halstedii* AJ-7 was then added to the culture. The culture was incubated at 28 °C for 24 h and the hyphal morphology was observed with a light microscope. (a) Normal mycelia of *P. capsici*; (b) abnormal swelling, curling, and branching of *P. capsici* mycelia grown with the high molecular fraction; (c) abnormal mycelia lysis of *P. capsici* mycelia grown with the low molecular fraction. The scale bar indicates 30 μm.

Table 4. Suppression of phytophthora blight by culture broth of *Streptomyces halstedii* AJ-7.

Growth (d)	Disease incidence (%)			
	Culture broth	Low molecular fraction[a]	High molecular fraction[a]	Not treated
3	0	0	2	15
6	0	0	5	32
9	0	0	13	49
12	3	2	16	70
15	4	3	23	99
18	5	4	38	100
21	10	6	46	100
24	18	9	60	100
27	20	12	68	100
30	28	20	72	100

[a]A culture broth of *S. halstedii* AJ-7 was fractionated into a low molecular fraction and a high molecular fraction, as described in Table 1. Red-pepper seeds were soaked in the culture broth, the low molecular fraction, and the high molecular fraction for 2 h. After being sown, red-pepper plug seedlings were grown in wet potting soil inoculated with the spore of the pathogen *Phytophtora capsici* (10^6 spores per g soil). During 30 d plant culture, the suppression of phytophthora blight by the culture broth was measured. The data are the mean of the effect on 105 seedlings.

Abnormal hyphal morphology of Phytophthora capsici *caused by culture broth of* S. halstedii *AJ-7*

An abnormal hyphal swelling, degradation, and lysis of mycelia were observed when *Phytophthora capsici* were grown with the high or low molecular fraction of *S. halstedii* AJ-7 (Figure 1). It is well known that chitinases lyze hyphae of some plant pathogenic fungi (De Boer *et al.* 1998, Patil *et al.* 2000). *S. halstedii* AJ-7 also produced extracellular chitinase (data not shown), suggesting that the abnormal hyphal morphology resulted from the chitinase activity produced from *S. halstedii* AJ-7.

Biocontrol of phytophthora blight by culture broth of S. halstedii *AJ-7*

As shown in Table 4, the phytophthora blight was significantly suppressed by the culture broth and the low molecular fraction of *S. halstedii* AJ-7 gave results that compared with those of the control red-peppers. The high molecular fraction did not significantly suppress the disease. These results indicate that *S. halstedii* AJ-7 is a good candidate to control *Phytophthora capsici*, which causes the phytophthora blight, and that the antifungal activity against the pathogen mainly originated from the low molecular fraction of the culture broth.

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