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Cellulolytic Enzymes of Antagonistic Actinomycetes against *Pythium aphanidermatum*, A Causative Agent of Seedling Damping off Disease in Plants

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Abstract

For the control of seedling damping off disease in plants caused by *Pythium aphanidermatum*, the actinomycetes were isolated from organic rich soil samples and screened for antagonistic activity against the pathogenic fungus. Only one isolate of actinomycetes demonstrated antagonistic effect against *P. aphanidermatum* by dual culture assay on Potato Dextrose Agar (PDA). The isolate S22 performed the best activity on fungal growth inhibition and was preliminary classified as *Streptomyces* sp. according to the aerial, substrate mycelial color and spore chain morphology. Since the cell wall polysaccharides of *P. aphanidermatum* comprised cellulose and β-glucans, the involvement of cellulolytic enzymes of the antagonistic *Streptomyces* isolate S22 on the fungal growth inhibition and mycelium destruction were investigated. The antagonistic assay between *Streptomyces* isolate S22 and *P. aphanidermatum* was tested on PDA amended with/without carboxymethyl cellulose (CMC). On PDA amended with CMC, the fungal growth was limited. The fungal mycelium could not penetrate into the agar zone that having the cellulase and other metabolic products produced by isolate S22. The damage of fungal structure as well as the releasing of reducing sugar from the mycelia of *P. aphanidermatum* after incubating in *Streptomyces* isolate S22 culture supernatant could be observed. This result indicates that the cellulolytic enzyme and metabolic products of *Streptomyces* isolate S22 may suppress the growth of *P. aphanidermatum*.

Keywords: antagonistic actinomycetes, *Pythium aphanidermatum*, hydrolytic enzymes, *Streptomyces*

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1. INTRODUCTION

Pythium aphanidermatum pre- and post-emergence damping-off and root rot of soybean are common in all soybean production areas and often contribute to a lower yield [1]. Diseases caused by this species are more common in regions with warm climates [2], but it has also been found to cause damage in greenhouse in colder regions [3]. The genus *Pythium* has been classified traditionally with other filamentous, coenocytic, sporangia-producing fungi as "Phycomyetes" [4]. The cell wall polysaccharides of *P. aphanidermatum* comprised 18% of cellulose and 82% of (1→3),(1→6)-β-d-glucans [5]. Many species of actinomycetes, particularly those belonging to the genus *Streptomyces,* are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi [6-9]. The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds [10] and extracellular hydrolytic enzymes [11]. *Streptomyces* are also known for their ability to produce fungal cell wall-degrading enzymes such as cellulases, hemicellulases, glucanases and chitinases [11, 12]. The role of these hydrolytic enzymes in antifungal activity and biocontrol ability of *Streptomyces* has been investigated [12, 13]. Many antibiotics produced by actinomycetes have been used directly or have been assumed to be responsible for the biocontrol potential of the producing strain [11, 14]

The objective of this study was to investigate the contribution of actinomycetes cellulolytic enzyme on the antagonistic activity against *Pythium aphanidermatum*.

2. MATERIALS AND METHODS

2.1 Isolation of actinomycetes from organic-rich soil samples

Five organic rich soil samples were collected from Ratchaburi province, Thailand. One gram of each organic-rich soil sample was transferred to 150 ml Nutrient Broth (NB) in 250 ml flask. The inoculated flasks were incubated in a rotary shaker at 150 rpm, 30°C, for 3-5 days. A 0.1 ml aliquot of suspension was spread-plated on Nutrient Agar (NA). The colonies that showed different morphology were selected and re-streaked on a new NA plate to obtain pure culture.

2.2 Screening for the antagonistic activity

Pythium aphanidermatum from the stock culture of the Department of Microbiology, KMUTT was used for testing the antagonistic activity of the isolated actinomycetes. The inhibition of mycelial growth of *P.aphanidermatum* by the actinomycetes isolates was tested using the dual culture technique on Potato Dextrose Agar (PDA). The conidia of the actinomycetes isolate were spot (0.6 cm diameter) inoculated on PDA at the distance of 1.5 cm from the edge of the Petri dish. Then the plates were incubated at 30°C for 5 days to allow growth and sporulation of the actinomycetes prior inoculation of *P.aphanidermatum* on to the plate. A 0.6 cm diameter PDA plug covered with active growing fungal mycelia was placed about 6 cm from the actinomycetes spot. The culture plates were incubated at 30°C for 7 days. The inhibition zone was determined by measuring the distance between the *P.aphanidermatum* and actinomycetes spots in the dual cultures.

2.3 Morphology and Taxonomy

The Morphology and taxonomy of the antagonistic actinomycetes isolates were determined using slide culture technique. The actinomycetes conidia were aseptically transferred on to a warm NA drop on a sterile glass slide in a moisture controlled Petri dish. The slide cultures were incubated at 30°C for 3 days before staining with carbon fuchsine and observing under a light microscope.

2.4 Cellulolytic activity of the antagonistic actinomycetes on solid medium

The isolated actinomycetes were spot inoculated on carboxymethly cellulose (CMC) agar (CMC 5 g/l; $(NH_4)_2SO_4$ 4 g/l; Na_2HPO_4 6 g/l; Yeast extract 1 g/l; $MgSO_4.7H_2O$ 0.2 g/l; agar 15 g/l) and incubated at 30°C for 5 days. To visualize the hydrolytic zone, the plates were flooded with an aqueous solution of 0.1% congo red for 15 min and washed with 1 M NaCl [13]. To indicate the cellulase activity of the organisms, diameters of clear zone around colonies on CMC agar were investigated.

2.5 The cellulolytic enzyme production of the selected antagonistic isolate

The cellulolytic enzyme production of the selected antagonistic isolate was studied in CMC liquid medium (CMC 5 g/l; $(NH_4)_2SO_4$ 4 g/l; Na_2HPO_4 6 g/l; Yeast extract 1 g/l; $MgSO_4.7H_2O$ 0.2 g/l). The culture supernatant was collected at 0, 1, 2, 3, 4, 5, 6, and 7 days. An aliquot of 0.5 ml of culture filtrate was added to the substrate (1% CMC in acetate buffer pH 6.0) and incubated at 37°C in water bath for 30 min. Appropriate control without of enzyme (culture supernatant) was simultaneously run. The reducing sugar produced in the reaction mixture was determined by dinitrosalicylic acid (DNS) method. An aliquot of 1.5 ml of dinitrosalicylic acid reagent was added to aliquots of the reaction mixture, heated at 100°C for 15 min. The absorbance of the reaction mixture at 540 nm was measured after cooling to room temperature. One unit of cellulase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per minute.

2.6 Testing for involvement of cellulolytic enzyme in antagonistic activity

The selected antagonistic isolates were streaked on 4 sides of a PDA plate amended with and without CMC and incubated at 30°C for 5 days to allow cellulolytic enzyme production. Then, a mycelium plug from 3 days old *P. aphanidermatum* was cut and transferred to antagonist-pregrown PDA plate. After incubation for 7 days, the fungal growth inhibitions on PDA plate amended with and without CMC were compared.

2.7 Enzymatic degradation of mycelium

To determine whether cellulase enzymes are involved in the antagonistic action of the selected isolate, the enzymatic degradation of mycelium of *P. aphanidermatum* were examined by the formation of reducing products of degradation of fungal cell wall components using DNS method. *P. aphanidermatum* was grown in Potato Dextrose Broth (PDB) for 3 days. Mycelium was collected and washed twice by centrifugation in a mixture (1:1) of sterile 0.9% NaCl and 0.025 M sodium phosphate buffer (pH6.0). Washed mycelium (1%, w/v) was incubated in a solution containing a complex of the selected isolate enzymes. The control samples contained the suspension of fungal mycelium and an equivalent volume of 25 mM sodium phosphate buffer (pH6.0). The mixture was incubated at 22±2°C for 24 h. Aliquots of the reaction mixture for the determination of reducing saccharides were taken every 6 h. The concentration of saccharides was calculated by the calibration plot, using glucose as a standard. The deformations of the *P. aphanidermatum* mycelium derived from the action of the antagonistic culture supernatant were observed under a light microscope.

3. RESULTS AND DISCUSSION

Twenty isolates of actinomycetes were recovered from the 5 organic-rich soil samples. Only the isolates S22 exhibited antagonistic activity against *P. aphanidermatum*. The antagonistic activity reflecting by zones of growth inhibition in the dual cultures are shown on Fig. 1.

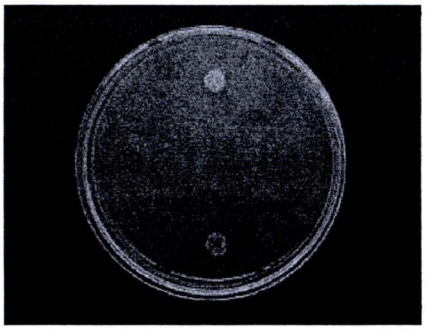

Fig. 1 Dual culture demonstrated the inhibition of *P. aphanidermatum* by the actinomycetes isolate S22

The isolate S22 grew on a range of culture media showing morphology typical of *Streptomycetes*, the colonies were slow growing, aerial and substrate mycelia of greenish yellow color (Fig.2a), spore chain with coiling (Fig.2b). The isolate S22 produced a large clear zone of hydrolysis on CMC agar. (Fig.3)

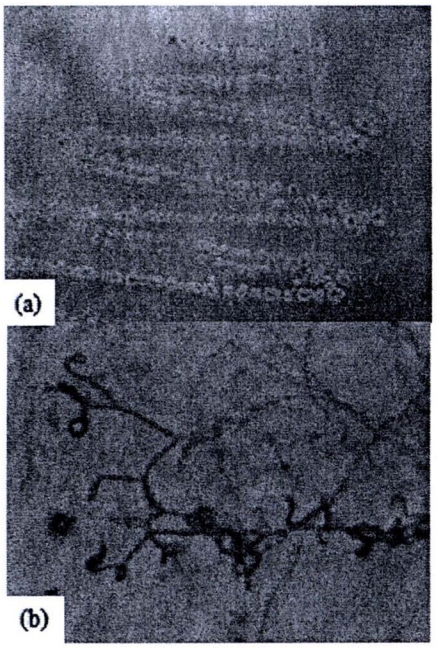

Fig. 2 Morphology of the isolate S22 colonies on PDA (a) and aerial mycelium with coiling spore chains (b)

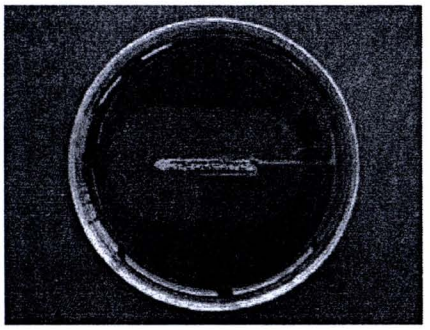

Fig. 3 Cellulolytic enzyme produced by the isolate S22 on CMC agar after staining with congo red

The cellulase activity of the isolate S22 on different days of incubation is shown in Fig.4. The isolate produced the highest cellulase activity at 4.88 units/ml on the third day.

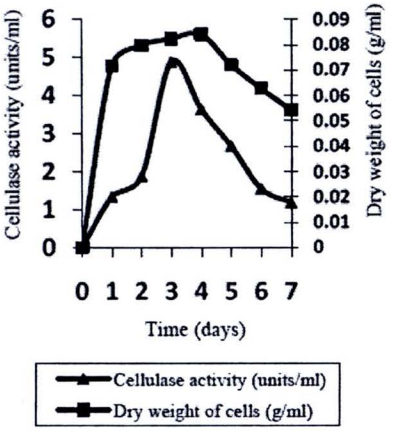

Fig.4 Cellululolytic enzyme production by the isolate S22

The *P. aphanidermatum* growth was limited by the isolate S22 on PDA amended with CMC compared to the fungal growth inhibition on PDA without CMC (Fig.5a, 5b). The cellulolytic enzyme produced by the antagonistic isolate S22 might have some effect on *P. aphanidermatum* growth inhibition. This effect may be due to the action of cellulolytic enzyme on the corresponding polymers of the fungal cell wall.

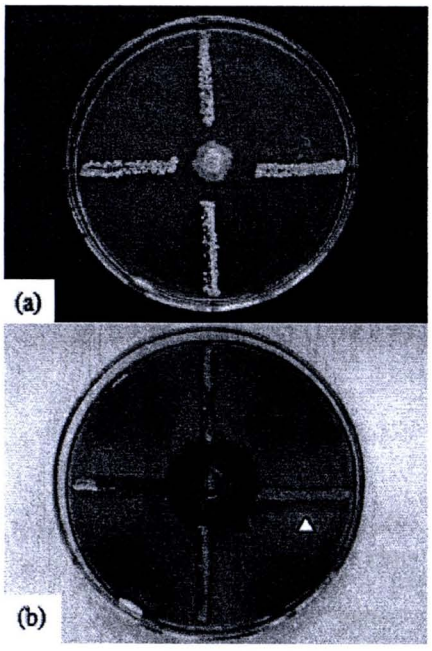

Fig. 5 Antagonist activity on PDA with CMC before (a), and after staining with congo red (b); the black arrow (▲) points at the fungal growth zone and the white arrow (△) points at the cellulolytic active zone (unstained)

Enzymatic degradation of *P. aphanidermatum* cell walls took places during incubation of the fungal mycelia with the antagonistic culture supernatant, which is accompanied by a rapid release of soluble hydrolytic products (Fig. 6).

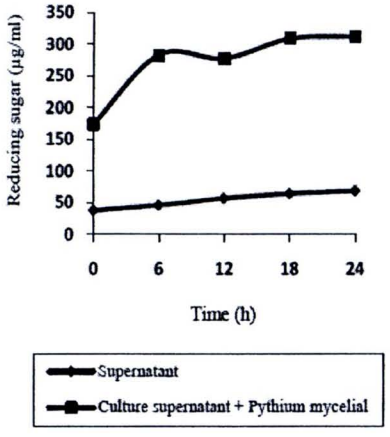

Fig.6 Dynamics of releasing of reducing saccharides (µg/ml) from *P. aphanidermatum* mycelium induced by cellulolytic enzyme of isolate S22.

The deformation and degradation of the cell wall of *P. aphanidermatum* mycelia incubated in the antagonistic culture supernatant were verified (Fig.7). The cellulolytic enzyme and metabolic products of the antagonistic isolate S22 may have participated in destroying the structure of *P. aphanidermatum*.

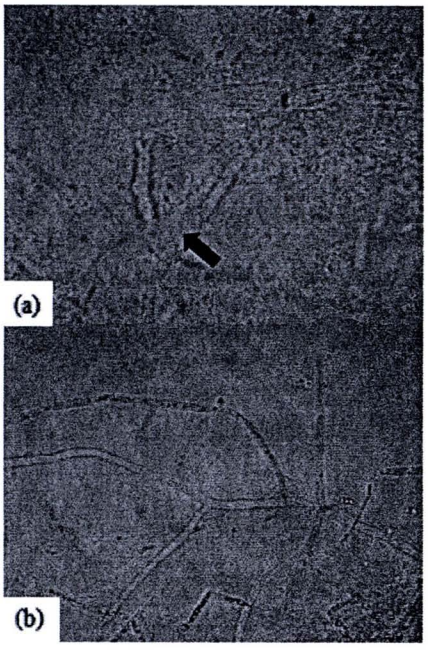

Fig 7 Mycelial of *P. aphanidermatum* after incubation for 24 h with: (a) the antagonistic culture supernatant, (b) 25 mM sodium phosphate buffer (pH 6.0)

4. CONCLUSION

The results of this study show that the *Streptomyces* isolate S22 could be a good biocontrol for *P. aphanidermatum*, a causative agent of seedling damping off and root rot diseases of plant due to their ability to synthesize antimicrobial substance and cellulolytic enzyme. Their extracellular enzymes and metabolic products can suppress the proliferation of *P. aphanidermatum*.

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