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Biocontrol of *Pythium aphanidermatum* by the cellulolytic actinomycetes *Streptomyces rubrolavendulae* S4

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ABSTRACT: *Streptomyces rubrolavendulae* (Yen) S4, isolated from a termite mound in Kanchanaburi, Thailand, was used to control the seedling damping-off disease of the horticultural plant Joseph's coat caused by *Pythium aphanidermatum* (Edson) Fitz. In a previous study, *S. rubrolavendulae* S4 was shown to strongly inhibit the growth of *P. aphanidermatum* on potato dextrose agar. This study investigated the modes of the antagonistic action of *S. rubrolavendulae* S4 on *P. aphanidermatum*. On carboxymethyl cellulose medium, *S. rubrolavendulae* S4 produced the highest cellulase activity of 65 U/ml in 5 days. The *S. rubrolavendulae* culture supernatant, with and without heat treatment, was tested for fungal growth inhibition using an agar well-diffusion method. The inhibitory effect was retained with the heat inactivated culture supernatant, indicating that the inhibition was derived from an antifungal compound rather than an enzyme produced by *S. rubrolavendulae* S4. The parasitic activity of *S. rubrolavendulae* S4 upon *P. aphanidermatum* was demonstrated by light and scanning electron micrographs. Joseph's coat (*Amaranthus tricolor*) seedlings were grown in peat mosses inoculated with *S. rubrolavendulae* S4 and *P. aphanidermatum*. In pots containing *S. rubrolavendulae* S4, the proportion of healthy seedlings was significantly increased. This was equivalent to the effect of the fungicide metalaxyl, and similar to the results from sterilized peat moss. Hence *S. rubrolavendulae* S4 can be used as an effective biological control agent in Joseph's coat cultivation.

KEYWORDS: antagonistic activity, fungal parasitism, scanning electron microscopy, Chinese spinach, tampala

INTRODUCTION

Damping-off is one of the most common horticultural disease in the greenhouse or nursery affecting germinating seeds and young seedlings[1]. In pre-emergence damping-off, the seeds wither or decay result in a sparse and patchy germination. In post-emergence damping-off, the pathogen infects the root-collar tissue and within days the seedlings become dark, rot nearly to the soil surface at the base of the stem, and then wilt and die. These diseases can spread quickly in a few days depending on the environmental conditions of the greenhouse or nursery[2]. *Pythium* spp., *Phytophthora* spp., *Fusarium* spp., *Rhizoctonia* spp., *Sclerotinia* spp., *Botrytis* spp., and *Alternaria* spp. are common soil saprophytic fungi that can cause damping-off diseases when the plant environmental conditions are suitable, i.e., excess moisture, low light, low temperature, and low pH of the growing medium[3].

Pythium is an oomycete, a fungal-like organism, commonly found in soil and water as a saprophyte called water mould. *Pythium* forms sporangia, sack-like structures that release swimming zoospores which survive in the soil, greenhouses and water by forming both oospores and chlamydospores. The genus *Pythium* has been widely reported as a plant pathogen in pre- and post-emergence plant seedlings[4]. Several *Pythium* species, including *P. aphanidermatum*, *P. irregular*, and *P. ultimum* are known to cause damping off, crown and root rot in cucumber, soya bean, chickpea, pepper, and tomato in greenhouses[5].

Greenhouses or nurseries usually use a chemical fungicide, such as metalaxyl, to reduce populations of *Pythium* in the soil[6]. Nowadays, biological control methods are sought as alternatives to chemical fungicides for controlling plant pathogens. Microbial antagonists have been widely applied for the biocontrol of fungal plant diseases such as *Streptomyces* spp., *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Trichoderma* spp[2,7]. Registered biocontrol products for *Pythium*-causing diseases of greenhouses in Denmark

are Mycostop (*S. griseoviridis*) and Supresivit (*Trichoderma harzianum*)[7]. The biological control mechanisms involved can be mutualism, protocooperation, commensalism, neutralism, competition, amensalism, parasitism, or predation[8].

Streptomyces is the largest genus within the actinomycetes group. These Gram-positive filamentous bacteria produce both substrate and aerial mycelium. Most *Streptomyces* produce spores with different characteristics that are used to identify the species of *Streptomyces*[9]. During sporulation, *Streptomyces* produces extracellular hydrolytic enzymes and antibiotics as secondary metabolites[10]. The interaction of *Streptomyces* with fungal pathogens is usually related to the production of cell wall-degrading enzymes such as cellulases, hemicellulases, chitinases, amylases, and glucanases[11]. Many species of *Streptomyces* are well known as antagonistic bacteria against several plant pathogenic fungi such as *S. halstedii*[12], *S. griseus*[13], and *S. lydicus* WYEC108[14]. In previous studies, *S. rubrolavendulae* S4 was shown to have effective antagonistic activity against *Fusarium oxysporum* and *Phytophthora infestans*[15,16]. In this study, the abilities of in vitro and in vivo antagonistic activities of *S. rubrolavendulae* S4 against *P. aphanidermatum*, a causative agent of seedling damping-off disease in Joseph's coat, were investigated.

MATERIALS AND METHODS

Microorganisms

S. rubrolavendulae S4, a cellulase producing actinomycetes, was isolated from a termite mound in Kanchanaburi province, Thailand and used as a biocontrol agent in this study.

Isolation and identification of plant pathogenic fungi

The infected Joseph's coat seedling pots were obtained from nurseries in Chiang Mai Province, Thailand. The stems of seedlings were washed with sterilized water to remove any excess of peat moss. Then, the infested plant parts were surface-sterilized using 5% (v/v) hypochlorite for 30 s, washed with sterilized water, and blot dried on sterile filter paper. The particles of stem and soil sample were placed onto Petri dishes with potato-dextrose-agar (PDA) and incubated at room temperature for 2–3 days. The mycelium of pathogenic fungi that grew out from the plant particles or soil sample was transferred onto a new potato-dextrose-agar plate. The isolated pathogenic fungi was identified based on the colony morphology (mycelium, oogonium and sporangium photographs) compared with the Van der Plaats-Niterink's photograph as a preliminary identification[17].

The partial ribosomal RNA gene, 18S ribosomal RNA gene was amplified by polymerase chain reaction with the universal primer pairs of internal transcribed spacer, ITS1 and ITS2 regions[18].The DNA sequencing analysis was carried out at Macrogen Sequencing Service, Korea using an ABI PRISM 3730XL capillary sequencer (Applied Biosystems, Foster City, CA, USA). The almost complete 18S ribosomal RNA gene was aligned with online database using BLAST search[19]. The evolutionary history was inferred using the Neighbour-Joining method[20]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa 110 analysed[21]. Phylogenetic analyses were conducted using MEGA4[22].

Antagonistic test by dual culture method

The pathogen-antagonist interaction was observed by using a dual culture technique on a solid medium. The *S. rubrolavendulae* S4 was streaked on PDA at the distance of 1.5 cm from the edge of the Petri dish. The plates were then incubated at 30 °C for 3 days to allow growth and sporulation of the *S. rubrolavendulae* S4. Afterwards a 4 mm diameter freshly growing mycelium plug of *P. aphanidermatum* was placed on the centre of a plate and incubated for another 3 days. The pathogen alone was used as a positive control and the experiments were repeated three times. A non-fungal growth area surrounding the *S. rubrolavendulae* S4 culture indicated antagonistic activity.

Growth and cellulase production

S. rubrolavendulae S4 cultured in CMC broth at 30 °C for 2 days was used for growth and cellulase production experiments. A 0.5 ml of cell suspension was transferred into 50 ml of carboxymethyl cellulose (CMC) culture medium (pH 6) (1.0 g/l of KH_2PO_4; 0.5 g/l $MgSO_4 \cdot 7H_2O$; 0.5 g/l NaCl; 0.01 g/l $FeSO_4 \cdot 7H_2O$; 0.01 g/l $MnSO_4 \cdot H_2O$; 0.3 g/l NH_4NO_3; and 10.0 g/l CMC) in a 250 ml Erlenmeyer flask and incubated at 30 °C in a rotary shaker at 200 rpm. The growth and cellulase activity were measured daily by using the drop plate[23] and dinitrosalicylic acid (DNS) methods. Cellulase activity was measured by mixing 0.5 ml of crude enzyme solution and 0.5 ml of 0.5% CMC in 50 mM phosphate buffer (pH 7.0). The reaction was then incubated at 50 °C in a shaking water bath at 100 rpm for 60 min and terminated by adding 3 ml of DNS reagent. The colour was developed by boiling the mixture for 5 min. Absorbance was measured by spectrophotometer (Hi-

tachi U-2010) at 540 nm. One unit (U) of enzyme activity was defined as the amount of enzyme which liberates 1 μmol of glucose per min under the above assay conditions.

Evaluation of fungal growth inhibition

The heat-inactivated extracellular enzyme of *S. rubrolavendulae* S4 was prepared by heating the supernatant from a 5 day culture in CMC medium at 100 °C for 10 min. The experiment was performed under strictly aseptic conditions. Wells were made in PDA plates with a sterile borer (4 mm diameter) and a mycelium plug of *P. aphanidermatum* was placed on the centre of the plate. A heated or non-heated supernatant sample of *S. rubrolavendulae* S4 (200 μl) was introduced into the wells. Sterile distilled water was used as a control. The plates were incubated at 37 °C for 2 days. All samples were tested in triplicate. Inhibition of fungal growth was determined.

Evaluation of the parasitic mechanism

The mycoparasitism of *P. aphanidermatum* cell wall by *S. rubrolavendulae* S4 was studied using light microscopy (CHS, Olympus optical Co. Ltd, Japan) and scanning electron microscopy (JEOL JSM-6610CV, Japan). Spores of *P. aphanidermatum* and the conidia of *S. rubrolavendulae* S4 were inoculated into a warm melted PDA drop on a sterile cover glass in a culture chamber at room temperature for 3–5 days and then observed by light microscopy. For making scanning electron micrographs, the cultures on the cover glasses were pre-fixed overnight in 2% $CH_2(CH_2CHO)_2$ in 0.1 M sodium cacodylate buffer pH 7.2–7.4 at 4 °C; washed three times with the same buffer for 15 min; post-fixed at 4 °C for 1 h in 1% OsO_4 in 0.1 M sodium cacodylate buffer; washed three times with 0.1 M sodium cacodylate buffer, pH 7.2–7.4 at 4 °C for 15 min; dehydrated in a graded series of ethanol and finally passed through a graded series of ethanol-amyl acetate. Samples in the amyl acetate were critical-point dried with Hitachi HCP-2 critical point dryer (HCP-2; Hitachi Co. Ltd., Japan); coated with Pt-Pd mixture for 4 min in Hitachi E-102 ion sputter (E-102; Hitachi Co. Ltd., Japan) and examined in a Hitachi S-510 scanning electron microscopy (JSM-6610CV; Japan Electron Optics Laboratory Co. Ltd.).

Evaluating the biocontrol of damping-off disease

The experiment was a 2×3 completely randomized factorial design with three replicates. Sterilized peat moss was used the growing medium in this study. The growing medium was prepared in two ways: artificially infested and non-infested with *P. aphanidermatum*. Infestation of peat moss with *P. aphanidermatum* was achieved by adding 50 plugs (4 mm diameter) of freshly-grown *P. aphanidermatum* into 250 g of steam-sterilized peat moss. Each preparation of the peat moss was separated into three treatments: (1) no pathogenic fungi, (2) *S. rubrolavendulae* S4 biocontrol treatment, and (3) Metalaxyl (Phyto-Q) fungicide treatment. The *S. rubrolavendulae* S4 biocontrol was applied at the rate of 10^6 CFU/g of peat mosses. Phyto-Q was used at the rate of 0.5 g/l according to the manufacturer's guidelines. Three replicates, each contains 50 Joseph's coat seedlings, were grown in the 6 treatments for 10 days. The percentage of disease suppression was calculated by counting the number of non-infested seedlings. Soil and dying seedlings were taken from the infected seedling pots for confirmation of the causative agent by direct plating on PDA.

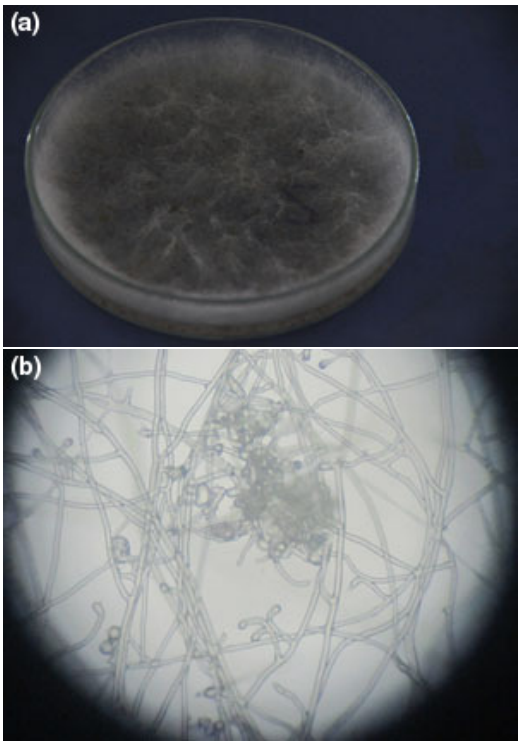

Fig. 1 *P. aphanidermatum*: (a) growth on potato dextrose agar; (b) aseptate mycelium under light microscope.

RESULTS

The *Pythium* sp. CS23 isolated from infected Joseph's coat seedling was identified as *P. aphanidermatum* based on the results of both morphological and molecular sequencing. The morphology of *P. aphanidermatum* on PDA and a photograph of aseptate mycelium of the fungi are shown in Fig. 1.

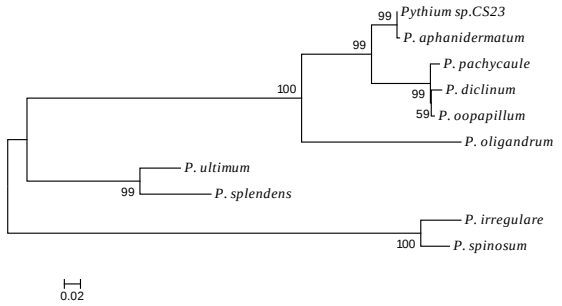

Fig. 2 The phylogenetic tree of 18S ribosomal RNA gene sequences between *Pythium* sp. CS23 and members of genus *Pythium*. Bar indicates 0.02 substitutions per nucleotide position.

The 825 bp sequence of 18S ribosomal RNA gene of *Pythium* sp. CS23 was found 100% identity to *Pythium aphanidermatum* isolate GH-A (EU327397). The phylogenetic tree of 18S ribosomal RNA gene sequences between *Pythium* sp. CS23 and members of genus *Pythium* is demonstrated in Fig. 2.

S. rubrolavendulae S4 was tested for antagonistic activity against *P. aphanidermatum* by mycelial growth inhibition on PDA. Good fungal growth inhibition was demonstrated using the dual culture technique (Fig. 3).

Growth and cellulase production of *S. rubrolavendulae* S4 in CMC medium were maximized at 7.85 $\log_{10}$ CFU/ml and 65.21 U/ml, respectively, in day 5 (Fig. 4). Enzyme production increased as growth increased. Since the major component of *P. aphanidermatum* cell wall is cellulose[24], the extracellular cellulase of *S. rubrolavendulae* S4 could have a potential use for destroying fungal mycelium.

Fungal growth inhibition by antifungal compounds and/or hydrolytic enzymes produced by the antagonist was tested using heat and non-heat inactivated culture supernatants of the antagonist. Both supernatants showed similar inhibitory effect on fungal growth (Fig. 5). The extracellular hydrolytic enzymes of the antagonist would have been destroyed by the high heat treatment used to prepare the samples. Hence, the fungal growth inhibitory effect achieved should be attributed to the activity of heat tolerant antifungal compounds produced by the antagonist. Nonetheless, hydrolytic enzymes may have a roll in the activity of the antagonist when controlling fungal mycelium.

When growing *S. rubrolavendulae* S4 with *P. aphanidermatum* in melted PDA on a cover glass, the penetration of mycelium of *S. rubrolavendulae* S4 into the colony of *P. aphanidermatum* was revealed under light microscopy (Fig. 6). The area where the *S. rubrolavendulae* S4 grew had a brown colour

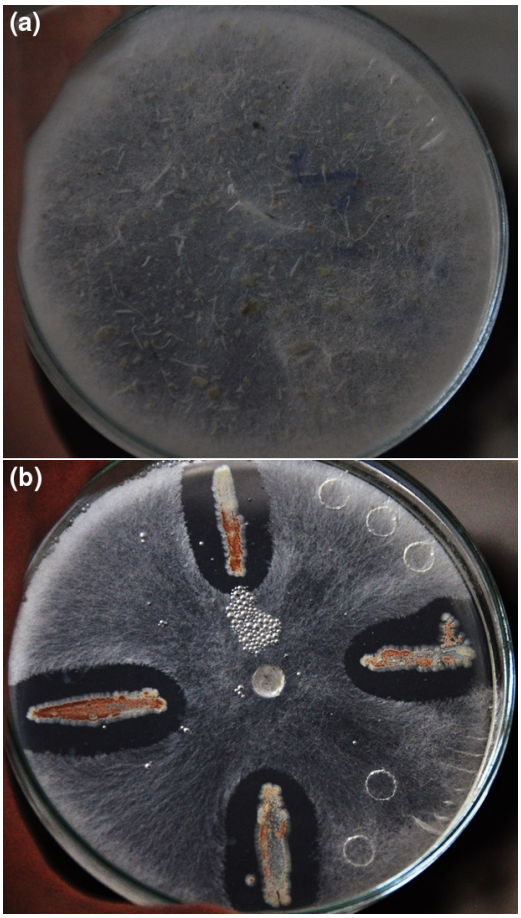

Fig. 3 Photographs of in vitro interactions between *S. rubrolavendulae* S4 and *P. aphanidermatum* in a dual culture on PDA: (a) control plate of *P. aphanidermatum* 6 days after inoculation; (b) four streaked lines of *S. rubrolavendulae* S4 against *P. aphanidermatum*.

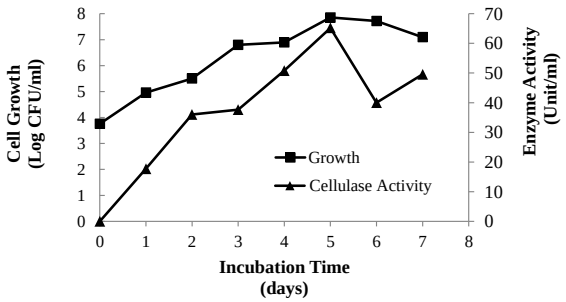

Fig. 4 The growth and cellulase activity of *S. rubrolavendulae* S4 cultured in CMC medium.

Fig. 5 The heat- and non-heat inactivated culture supernatant of *S. rubrolavendulae* S4 showed inhibitory effect on *P. aphanidermatum* in growth. Two days after incubation, *P. aphanidermatum* photographed from the bottom.

Table 1 Biological and chemical control of Joseph's coat damping-off disease caused by *P. aphanidermatum*.

Treatment	Infected (%)
Non-infected peat moss	
Control	87.5[b]
S. rubrolavendulae S4	81.8[b]
Phyto-Q	94.0[b]
Infected peat moss (*P. aphanidermatum*)	
P. aphanidermatum	46.9[a]
P. aphanidermatum + *S. rubrolavendulae* S4	74.0[b]
P. aphanidermatum + Phyto-Q	81.3[b]

Means within a column with the same letter are not significantly different ($p > 0.05$).

indicating that spore production was occurring. The brown colour was observed on the mycelia mass of *P. aphanidermatum* indicating the likely production of extracellular enzymes and antibiological compounds. The degradation of the fungal mycelium by the antagonist was also observed (Fig. 7a,b). The complete colonized *S. rubrolavendulae* S4 on *P. aphanidermatum* mycelium was verified by scanning electron microscopy (Fig. 7c,d). The results confirm that *S. rubrolavendulae* S4 could colonize on *P. aphanidermatum* mycelium.

Symptoms of damping-off were observed within 5–10 days of Joseph's coat seedlings being planted into the inoculated peat moss growing medium (Table 1). In the sterile growing medium, the proportion of healthy, non-infected seedlings was greater than 82% in all treatments. When seedlings were grown on peat moss contaminated with *P. aphanidermatum*, the proportion of non-infected seedlings was only 47% which was significantly different from the control treatment (88%). Treatment with *S. rubrolavendulae* S4, as a biological control agent, markedly suppressed damping-off disease to levels similar to those achieved with the control and with the fungicide treatment. These results indicate that *S. rubrolavendulae* S4 is an effective biocontrol against *P. aphanidermatum*.

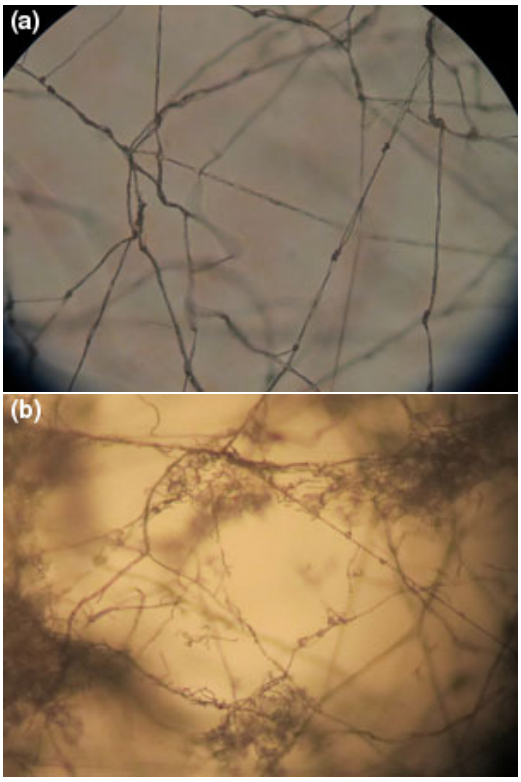

Fig. 6 Light micrographs of colonized *S. rubrolavendulae* S4 on *P. aphanidermatum* mycelium: (a) normal mycelium of *P. aphanidermatum* on PDA; (b) micrograph of smaller and thinner *S. rubrolavendulae* S4 mycelium growing along *P. aphanidermatum* mycelium. Arrows indicate mycelium of *S. rubrolavendulae* S4.

DISCUSSION

Joseph's coat seedlings are traditionally produced in plastic pots using peat moss as a planting material. It takes only 7–10 days before the seedling are sold to customers. Chemical fungicide treatments are unsuitable for such a short time of cultivation, since the residues of these hazardous chemicals remain

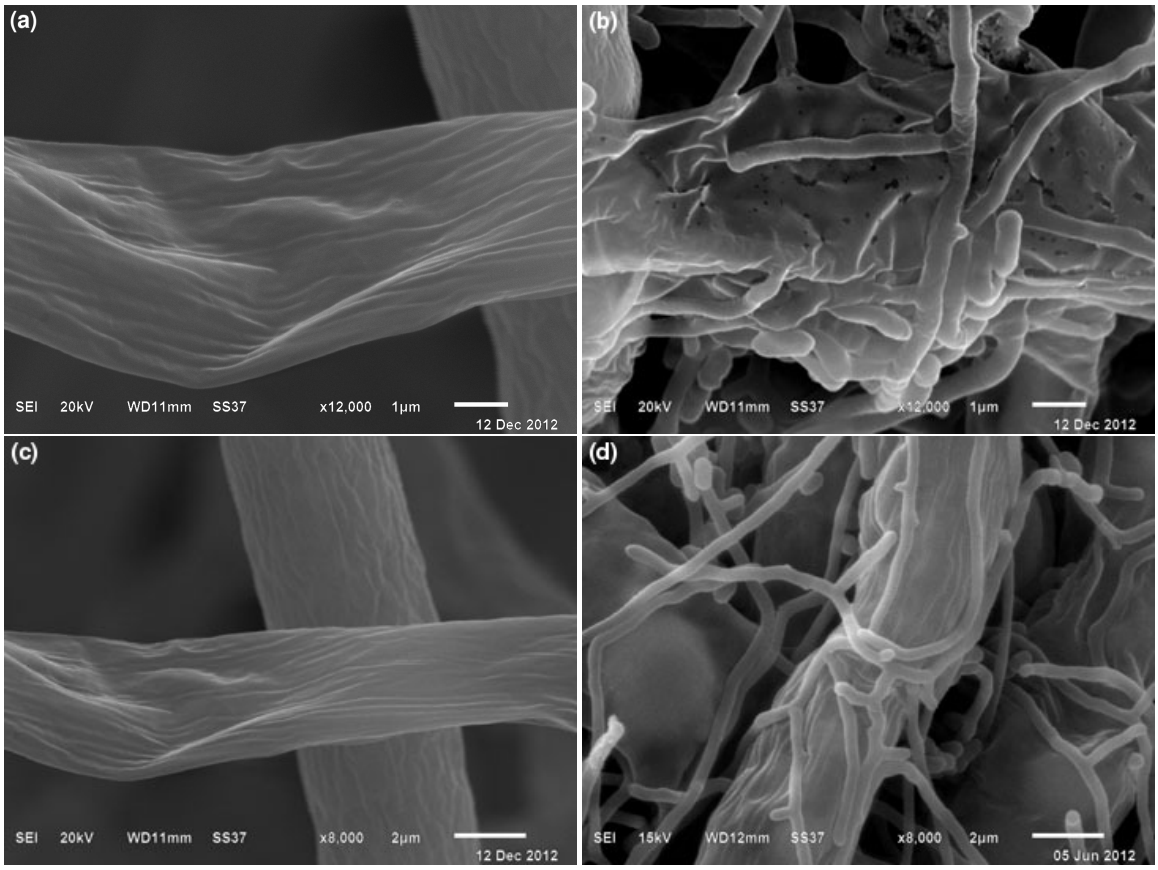

Fig. 7 Scanning electron micrographs of the deformation of the fungal mycelium by the antagonist 5 days after co-culturing: (a) mycelium of *P. aphanidermatum* (bar = 1 µm); (b) mycelium pores of *P. aphanidermatum* caused by *S. rubrolavendulae* S4; (c) mycelium of *P. aphanidermatum* (bar = 2 µm); (d) *S. rubrolavendulae* S4 hyphae growing on mycelium of *P. aphanidermatum*.

on the edible parts of the plants. Hence biological control methods would be a better alternative approach under such production methods. In recent studies, *Streptomyces* species have been reported to be a commercial biocontrol agent (BCA) against *Pythium* species. These species include *S. griseoviridis* (Mycostop)[7]and *S. lydicus* WYEC108 (Actino-Iron)[14]. *S. griseoviridis* (Mycostop) has been shown to produce enzymes that break down the cell wall of *Fusarium* spp., *Phytophthora* spp., and other disease-causing pathogens[7]. It has also been shown that *S. lydicus* WYEC108 could inhibit *P. ultimum* growth[14].

In this study, growth of *P. aphanidermatum*, isolated from collapsed Joseph's coat seedlings, could be suppressed by *S. rubrolavendulae* S4. *S. rubrolavendulae* S4 appeared to alter the structure of *P. aphanidermatum* mycelia by lysing hyphal tips that caused swelling and abnormal branching of hyphae. In this experiment, the degradation of *P. aphanidermatum* mycelium was demonstrated by electron micrographs of co-culture of the antagonist and the pathogen. The *S. rubrolavendulae* S4 cellulase was likely to be the agent destroying of the fungal mycelium. Several studies have suggested that the biological agents (BCAs) should be introduced early in systems for colonization in the rhizosphere before planting crops[25]. In the plant protection experiment reported here, the antagonistic *S. rubrolavendulae* S4 protected seedlings from damping-off disease when the antagonist was colonized in the planting material before artificial infection of *P. aphanidermatum* occurred.

The cellulolytic *S. rubrolavendulae* S4 isolated from Amphoe Si-Sawat, Kanchanaburi province had high cellulase activity. In the current study, the *S. rubrolavendulae* S4 was shown to inhibit growth and colonize on *P. aphanidermatum*, a causative agent of seedling damping-off disease of Joseph's coat. The mycoparasitism of *S. rubrolavendulae* S4 against

P. aphanidermatum revealed that *S. rubrolavendulae* S4 could be a good biocontrol agent in greenhouse production and in plant nurseries, especially those involved with Joseph's coat horticulture.

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REFERENCES

1. Hulvey J, Gobena D, Finley L, Lamour K (2010) Co-occurrence and genotypic distribution of *Phytophthora* species recovered from watersheds and plant nurseries of eastern Tennessee. *Mycologia* **102**, 1127–33.
2. Postma J, Geraats BP, Pastoor R, van Elsas JD (2005) Characterization of the microbial community involved in the suppression of *Pythium aphanidermatum* in cucumber grown on rockwool. *Phytopathology* **95**, 808–18.
3. Zad SJ, Koshnevice M (2001) Damping-off in conifer seedling nurseries in Noshahr and Kelardasht. *Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet* **66**, 91–4.
4. Sánchez J, Gallego E (2002) Fitopatogenicidad de Pythium spp. presentes en el agua de riego del Poniente almeriense (sureste de España). *Rev Iberoam Micología* **19**, 177–80.
5. Chen MH, Nelson EB (2008) Seed-colonizing microbes from municipal biosolids compost suppress *Pythium ultimum* damping-off on different plant species. *Phytopathology* **98**, 1012–8.
6. Mocioni M, Titone P, Garibaldi A, Gullino ML (2003) Efficacy of different fungicides against Rhizoctonia brown patch and Pythium blight on turfgrass in Italy. *Comm Agr Appl Biol Sci* **68**, 511–7.
7. Hansen VM, Winding A, Madsen AM (2010) Exposure to bioaerosols during the growth season of tomatoes in an organic greenhouse using Supresivit (*Trichoderma harzianum*) and Mycostop (*Streptomyces griseoviridis*). *Appl Environ Microbiol* **76**, 5874–81.
8. Janisiewicz WJ, Tworkoski TJ, Sharer C (2000) Characterizing the mechanism of biological control of postharvest diseases on fruits with a simple method to study competition for nutrients. *Phytopathology* **90**, 1196–200.
9. Li J, Tian XP, Zhu TJ, Yang LL, Li WJ (2011) *Streptomyces fildesensis* sp. nov., a novel streptomycete isolated from Antarctic soil. *Antonie Leeuwenhoek* **100**, 537–43.
10. Elleuch L, Shaaban M, Smaoui S, Mellouli L, Karray-Rebai I, Fourati-Ben Fguira L, Shaaban KA, Laatsch H (2010) Bioactive secondary metabolites from a new terrestrial *Streptomyces* sp. TN262. *Appl Biochem Biotechnol* **162**, 579–93.
11. Chater KF, Biro S, Lee KJ, Palmer T, Schrempf H (2010) The complex extracellular biology of Streptomyces. *FEMS Microbiol Rev* **34**, 171–98.
12. Joo GJ (2005) Purification and characterization of an extracellular chitinase from the antifungal biocontrol agent *Streptomyces halstedii*. *Biotechnol Lett* **27**, 1483–6.
13. Rabeeth M, Anitha A, Srikanth G (2011) Purification of an antifungal endochitinase from a potential biocontrol Agent *Streptomyces griseus*. *Pakistan J Biol Sci* **14**, 788–97.
14. Yuan WM, Crawford DL (1995) Characterization of *Streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. *Appl Environ Microbiol* **61**, 3119–28.
15. Loliam B, Chaiyanan S, Morinaga T (2010) Chitinase effectivity of Antagonistic Streptomyces sp against *Fusarium oxysporum*. In: *International Conference for a Sustainable Greater Mekong Subregion*, Bangkok.
16. Loliam B, Morinaga T, Chaiyanan S (2012) *Biocontrol of Phytophthora infestans, Fungal Pathogen of Seedling Damping-off Disease in Economic Plant Nursery*, Hindawi, New York, pp 1–6.
17. Plaats-Niterink AJVD (1981) *Monograph of the Genus Pythium*, Studies in Mycology No. 21, Centraalbureau voor Schimmelcultures, Baarn.
18. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Michael AJ, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR Protocols: A Guide to the Methods and Applications*, Academic Press, New York, pp 315–22.
19. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–402.
20. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–25.
21. Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783–91.
22. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–9.
23. Herigstad B, Hamilton M, Heersink J (2001) How to optimize the drop plate method for enumerating bacteria. *J Microbiol Meth* **44**, 121–9.
24. Manocha MS, Colvin JR (1968) Structure of the cell wall of *Pythium debaryanum*. *J Bacteriol* **95**, 1140–52.
25. Xu XM, Jeffries P, Pautasso M, Jeger MJ (2011) Combined use of biocontrol agents to manage plant diseases in theory and practice. *Phytopathology* **101**, 1024–31.