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Potential of a Soil-Borne *Streptomyces hygroscopicus* for Biocontrol of Anthracnose Disease Caused by *Colletotrichum gloeosporioides* in Orchid

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Abstract: The aims of this study are to isolate the antagonist from rhizosphere soil and evaluate its potential for biocontrol of anthracnose disease in orchid. The potential microbial antagonist, designated SRA14, was isolated and identified as *Streptomyces hygroscopicus*. Cell-free culture filtrates of *Streptomyces hygroscopicus* SRA14 inhibited the growth of *Colletotrichum gloeosporioides*. The percentage of growth inhibition by the stationary culture filtrate was significantly higher than that of exponential-culture filtrate. Additionally, morphological changes such as hyphal swelling and abnormal shapes were observed in fungi grown on potato dextrose agar that contained the culture filtrates. Application of culture filtrates was able to prevent the expression of anthracnose disease on orchid, indicating that disease inhibition was due to antifungal metabolites in the culture filtrates. No significant prevention of anthracnose development was observed in the stationary culture filtrate and mancozeb-treated leaves. Present data suggested the stationary culture filtrate of SRA14 can be used as biofungicide for control of anthracnose diseases in *Dendrobium* orchid.

Key words: Antifungal metabolites, actinomycetes, plant disease, biocontrol agent

INTRODUCTION

Dendrobium orchid is one of the important economical cut-flower plants in many countries. However, orchid cultivation has faced a lot of problems, particularly fungal diseases. *Colletotrichum gloeosporioides* (Penz.) causes anthracnose disease in a wide variety of agricultural crops worldwide (Bautista-Banos *et al.*, 2003; Gamagae *et al.*, 2003). Chemical fungicides are extensively used in current agriculture. However, excessive use of chemical fungicides in agriculture has led to deteriorating human health, environmental pollution and development of pathogen resistance to fungicide. Microbial antagonists are widely used for the biocontrol of fungal plant diseases due to lack of induction of pathogen resistance and reduction of chemical fungicide residues in the environment. Antagonism may be accomplished by competition, parasitism, antibiosis, lysis or by a combination of these modes of action (Chernin and Chet, 2002).

Many species of *Streptomyces*, are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi e.g., *Gauemannomyces graminis* var. *tritici* (Coombs *et al.*, 2004), *Phytophthora capsici*

(Joo, 2005), *Fusarium oxysporum* f. sp. *cubense* (Cao *et al.*, 2005), *Sclerotium rolfsii* (Errakhi *et al.*, 2007). The antagonistic potentials of *Streptomyces* to pathogenic fungi involved the production of extracellular hydrolytic enzymes and moreover, secondary antifungal compounds were also reported by Trejo-Estrada *et al.* (1998a), El-Tarabily *et al.* (2000) and Ouhdouch *et al.* (2001). However, data related to the potential of the extracellular metabolites of *Streptomyces* strains to control the anthracnose disease caused by *C. gloeosporioides* are limited. In this study, we report the powerful *Streptomyces* strain that is able to control the anthracnose disease caused by *C. gloeosporioides* in *Dendrobium* orchid. The antifungal metabolites produced by this antagonist could be applied for their future use as a biofungicide for the control of anthracnose disease in several agricultural plants.

Antifungal activity of cell-free culture filtrates produced by a potent antagonistic streptomyces against *C. gloeosporioides*: Twenty-four random rhizosphere soil samples were collected from paddy fields and orchards in the central part of Thailand. The soil suspension was spread onto starch-casein-agar plates and incubated at

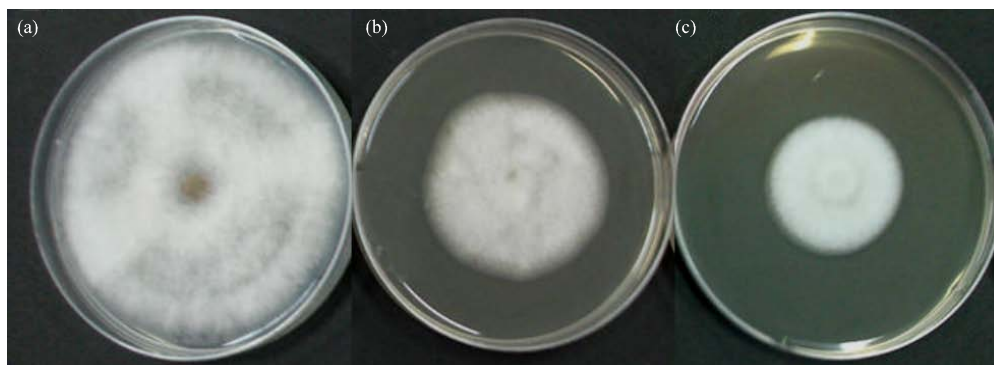


Fig. 1: Antifungal activity of cell-free culture filtrates of the SRA14 against *C. gloeosporioides* on PDA plates. *In vitro* antagonism of the strain SRA14 to *C. gloeosporioides* was evaluated on PDA plates. The strain SRA14 was grown on PDA plate amended with either 20% (v/v) exponential (b) or stationary (c) culture filtrates. Control culture of *C. gloeosporioides* was cultivated on culture filtrate-free PDA (a). Growth inhibitory activity was observed after 6 days of incubation in comparison to the controls

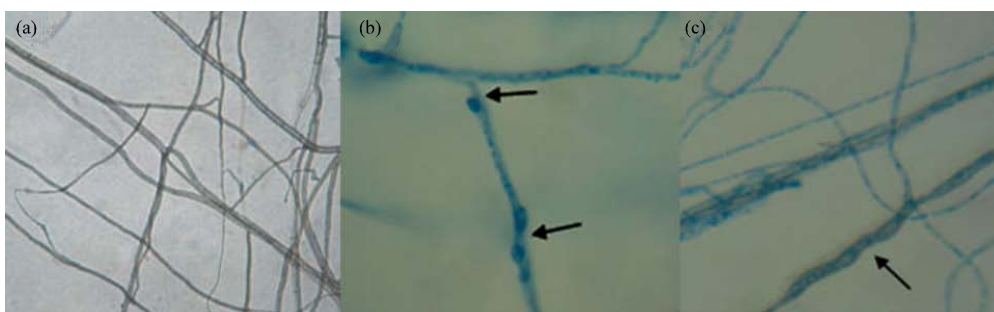


Fig. 2: Effects of cell-free culture filtrates on the hyphal morphology of *C. gloeosporioides*. Hyphal morphology of 3 day-old *C. gloeosporioides* grown on PDA amended with 20% (v/v) exponential (b) and stationary (c) culture filtrates was observed under light microscopes (40X) compared with the fungal hyphal cultured on PDA plate (a)

28°C for 12-14 days. Colonies of *Streptomyces* on the agar plates were picked on the basis of their morphological characteristics and purified on ISP-2 agar (Shirling and Gottlieb, 1966). All the isolates were screened for their *in vitro* antagonism against *C. gloeosporioides* by dual culture assay, according to the modified method of Crawford *et al.* (1993). The SRA14 had a strong antagonistic activity to *C. gloeosporioides*. Analysis of the 16S rDNA gene sequences showed that the SRA14 is closely related to *Streptomyces hygroscopicus* (98% similarity) with GenBank database accession number AB184760. It has been reported that *Streptomyces violaceusniger* G10 showed a strong antagonism toward *F. oxysporum* f. sp. *ubense* by producing extracellular antifungal metabolites (Getha and Vikineswary, 2002). Fermentation broth of the SRA14 cultured in ISP-2 broth (Shirling and Gottlieb, 1966) was collected at 1 and 3 days to obtain the exponential phase and stationary phase cultures, respectively. The cell-free supernatant was filtered aseptically through a sterile membrane with

0.45 μ m pore size. Culture supernatant was added to Potato Dextrose Agar (PDA) to yield a final concentration of 20% (v/v). The radial growth of *C. gloeosporioides* was inhibited by both the 20% (v/v) exponential (Fig. 1b) and stationary (Fig. 1c) culture filtrates in comparison with the control culture (Fig. 1a). The results implied that the culture filtrates of the SRA14 contain the antifungal metabolite(s).

The finding showed that extracellular metabolites in the culture filtrates of the SRA14 inhibited the growth of pathogenic fungi prompted the investigation of the effects of culture filtrates on the fungal hyphal structure. Light microscope investigation revealed that growth inhibition of *C. gloeosporioides* as a response to the exponential (Fig. 2b) and stationary (Fig. 2c) culture filtrates was accompanied by marked cellular changes including hyphal swelling, distortion and bulbous roundedness on hyphal structure. None of these abnormal shapes were observed in the control experiments (Fig. 2a). Abnormal hyphal structures of the

inhibited fungal hyphae resulting from diffusible antifungal secondary compounds have been previously reported by Getha and Vikineswary (2002) and Taechowisan *et al.* (2005). In addition, the antifungal mechanism of antagonists has been attributed to the action of hydrolytic enzymes such as chitinase, β -1,3-glucanase and protease (De Boer *et al.*, 1998; Wang *et al.*, 1999, 2002).

Production of extracellular chitinase enzyme involved in the antifungal potential of *S. hygroscopicus* SRA14:

As stated in many previous reports, the production of chitinase enzyme by *Streptomyces* was related to fungal growth inhibition and the biological control of fungal pathogens was possible because of the ability of *Streptomyces* to degrade fungal cell walls (Mahadevan and Crawford, 1997, 1999; El-Tarabily *et al.*, 2000; Mukherjee and Sen, 2006). The SRA14 was grown in ISP-2 broth and collected cell-free supernatants for chitinase determination. The estimation of chitinase activity was carried out by the procedure described by Wang *et al.* (2002) using colloidal chitin as substrate. The SRA14 produced relatively high levels of chitinase (5.2 U mg⁻¹ protein) at day 1 of the incubation period. The level of chitinase activity was sharply increased during the exponential phase and dramatically declined when the cells entered the stationary phase (Data not shown). According to Joo (2005), *Streptomyces halstedii* AJ-7 produces extracellular chitinase and causes abnormal hyphal morphology. The culture filtrate of *Pseudomonas aeruginosa* K-187 causes growth aberration, hyphal swelling and lysis of many fungi due to its high content of chitinase enzyme (Wang *et al.*, 1999). The antifungal potential of the exponential culture filtrate was probably related to the increased production of chitinase enzyme during the exponential phase. It should be noted that one of the possible antifungal mechanisms of the SRA14 may be associated with the production of extracellular chitinase enzyme.

Stationary culture filtrate possessed a higher antifungal potential than the exponential culture filtrate:

Growth inhibitory experiment was then performed to find out whether exponential or stationary culture filtrates play the major role in the fungal growth inhibition. Culture supernatants from both the growth phases of the SRA14 were collected and aseptically added to Potato Dextrose Broth (PDB) to yield a final concentration of 20% (v/v). Spore suspension (10⁴ spores mL⁻¹) of *C. gloeosporioides* was inoculated into PDB medium incubated on incubator shaker at 120 rpm, 28°C for 1, 2, 3, 5 and 7 day, respectively. The fungal growth monitored

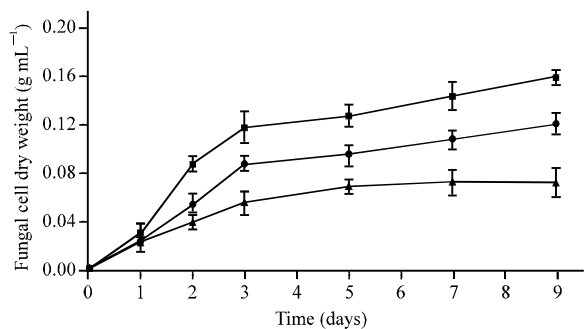


Fig. 3: Antifungal effects of cell-free culture filtrates obtained from the strain SRA14 as observed by the submerged growth inhibition of *C. gloeosporioides*. *C. gloeosporioides* was grown on PDB, amended with either 20% (v/v) exponential (●) or stationary (▲) culture filtrates. Control culture of *C. gloeosporioides* (■) was cultivated on culture filtrate-free PDB. Mycelial growth of the fungus was monitored by measuring the cell dry weight at 1 day intervals. The experiment was independently repeated at least three times and representative data are shown

by dry weight determination. The result found that the submerged growth of *C. gloeosporioides* was inhibited by both the 20% (v/v) exponential and stationary culture filtrates in comparison with the control culture (Fig. 3). These results indicated that fungal growth suppression resulted from the presence of extracellular antifungal metabolites in culture filtrates. The percentages of the submerged growth inhibition of *C. gloeosporioides* by the exponential and stationary culture filtrates on the day 3 of incubation were 26.58 and 53.77%, respectively. El-Abyad *et al.* (1993) reported that the culture filtrate of either *Streptomyces pulcher* or *Streptomyces canescens* at a concentration of 80% (v/v) inhibited the mycelial growth of *F. oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum* and *Alternaria solani*.

The percentage of growth inhibition by the stationary culture filtrate was significantly higher than that of exponential culture filtrate. These findings imply that the antifungal potential of the culture filtrates is not solely due to the increased chitinase production, as shown by the increase in percentage of fungal growth inhibition by the stationary culture filtrate. There is a possibility that the increased antifungal activity against *C. gloeosporioides* by the stationary culture filtrate of the SRA14 is a consequence of the production of extracellular secondary antifungal compound(s). The production of secondary antifungal compound(s) as antibiotics has been already reported in many species of *Streptomyces*



Fig. 4: Biocontrol of anthracnose disease on *Dendrobium* leaves by cell-free culture filtrates of the SRA14. Orchid leaves were treated with different treatments including, (T₁) no treatment as negative control, (T₂) *C. gloeosporioides* as positive control, (T₃) 200 μ L of 1 mg mL⁻¹ of mancozeb as chemical fungicide control, (T₄) 200 μ L of the exponential culture filtrates, (T₅) 200 μ L of the stationary culture filtrates and (T₆) 200 μ L of overnight culture (10⁵ spores mL⁻¹) of the SRA14. The 100 μ L of spore suspension (10⁴ spores mL⁻¹) of *C. gloeosporioides* was placed on abaxial surface of each orchid leaf in all treatments, except T₁. The visible symptom appearance of all orchid leaves was observed for 5 days after fungal infection

(Xiao *et al.*, 2002; Fguira *et al.*, 2005; Taechowisan *et al.*, 2005). The degree of fungal growth inhibition corresponded to the increase in concentration of the culture filtrate (data not shown). The growth inhibitory effects of culture filtrates decreased with the extension of fungal incubation period. Other previous studies reported that the decreases in the degree of growth inhibition were associated with the increases in the incubation period of the fungal culture (El-Abyad *et al.*, 1993; Chang *et al.*, 2007).

Preventive application of culture filtrates from antagonistic rhizobacteria on anthracnose disease expression in *Dendrobium* leaves: According to the *in vitro* antagonism against *C. gloeosporioides*, *S. hygroscopicus* SRA14 was evaluated for *in vivo* biocontrol of anthracnose disease in 1 year-old *Dendrobium* plants. The experiment used a Completely Randomized Design (CRD) which was divided into 6 treatments with 5 replications. The abaxial surface of orchid leaf was scratched with a sterile needle to make a little wound. Then, 100 μ L of spore suspension (10⁴ spores mL⁻¹) of *C. gloeosporioides* was applied on abaxial surface of each orchid leaf in all treatments, except negative control treatment (T₁). The sizes of visible lesion on orchid leaves were measured for 5 days after fungal infection. The results in greenhouse study showed that the Disease Severity Index (DSI) was significantly

($p \leq 0.05$) reduced on orchid leaflets treated with the exponential (T₄), stationary (T₅) culture filtrates and cell culture of the SRA14 (T₆) compared with the absence of the biocontrol agent (T₂) (Fig. 4). Application of the culture filtrates of the SRA14 inhibited anthracnose disease on orchid leaves, indicating that suppression was due to antifungal compounds in the culture filtrate. The stationary culture filtrate was more suppress the symptom expression than that of exponential culture filtrate. There was no significantly different in the control of anthracnose development on orchid leaves between the stationary culture filtrate and chemical fungicide treatment (T₃). The results indicated that the stationary culture filtrate controlled anthracnose disease as effectively as the chemical control. Several studies have demonstrated that many of the secondary compounds produced by *Streptomyces* strains have a broad spectrum activity against plant fungal pathogens (Trejo-Estrada *et al.*, 1998; Fguira *et al.*, 2005; Taechowisan *et al.*, 2005). Antibiotic production by *S. hygroscopicus* can inhibit a broad range of fungal pathogens e.g., *Rhizoctonia solani*, *Pythium ultimum*, *F. oxysporum* and *Sclerotinia homeocarpa* (Rothrock and Gottlieb, 1984; Chamberlain and Crawford, 1999). In conclusion, there is good potential for using the stationary culture filtrate of *S. hygroscopicus* SAR14 to as biofungicide to control the anthracnose disease in orchid cultivation.

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