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Evaluation of Crude Extract Substances from *Streptomyces* spp for Controlling *Colletotrichum gloeosporioides* Caused Anthracnose of Chili

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Abstract Anthracnose of chili caused by *Colletotrichum gloeosporioides*, is a major problem among important economic crops. In the present study, 34 isolates of *Streptomyces* spp. were used to determine the inhibition of *C. gloeosporioides* growth using dual culture test. The results showed 19 isolates of *Streptomyces* spp. had the percent inhibition of *C. gloeosporioides* growth and the highest percent inhibition of radial growth was *Streptomyces* isolate CH15. In addition, the percent inhibition of radial growth was *Streptomyces* isolate CH15 was not significantly different from 0.75 mg/ml benomyl. Then, the ethyl acetate extract of *Streptomyces* isolate CH15 at concentration 87.7 mg/ml in 5% (V/V) DMSO was tested for inhibiting *C. gloeosporioides* by the disc diffusion method. The results showed that the efficiency of the ethyl acetate extract of isolate CH15 for controlling *C. gloeosporioides* was not significantly different from 0.75 mg/mL benomyl.

Keywords: *Colletotrichum gloeosporioides*, crude extract substances, *Streptomyces* spp.

Introduction

Chili (*Capsicum annuum* L.) is an indispensable spice and vegetable crops used as a basis of its high consumption, nutritional and cash value to the farmers and consumers in the tropical and subtropical area. Chili fruits are consumed as fresh, dried or processed products, table purpose, spice and condiments. Chemical compounds of chili fruit comprise steam volatile oils, fatty oils, capsaicinoids, carotenoids, vitamins, protein, fibre and minerals. Moreover, the fresh fruit of chili contains more vitamin C and more vitamin A than many fruits (Bosland and Votava, 2003). Chili is one of the most important economic vegetables in Thailand. The cultivation of chili spreads every provinces of Thailand. The total planting area and the quantity of the product for chili production in 2006/2007 were 75,955 ha and 333,672 ton (Milerue *et al*, 2016). Many chili varieties are severely damaged by anthracnose

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which may cause yield losses of up to 50% (Pakdeevaraporn *et al.*, 2005). Typical anthracnose symptoms Anthracnose disease caused by *Colletotrichum gloeosporioides* [telemorph *Glomerella cingulata* (Stoneman) (Spauld and Schrenk, 1903)] is one of the chief hindrances for chili production (Moe Oo and Oh, 2016). Anthracnose symptoms on chili fruit include sunken necrotic tissues, water-soaked and concentric markings. In addition, fruits showing blemishes loose marketability. The attack symptoms in flower bud, the tops of the affected branches wither and turn brown (Moe Oo and Oh, 2016).

Control of chili anthracnose from *C. gloeosporioides* has been accomplished mainly with chemical fungicides, such as benomyl, maneb, chlorothalonil and mancozeb (Palaniyandi *et al.*, 2011). However, chemical agents cannot completely remove this chili anthracnose from *C. gloeosporioides* effectively and sustainably. Therefore, biological control of diseases and pests of crops using microbial antagonists has been consequently considered as an alternative method, which could reduce expenses and is not toxic to the environment (Moenne-Loccoz *et al.*, 2001). Several researches using a variety of microbial antagonists are being studied extensively with many different plant diseases. Actinomycetes are known producers of several secondary metabolites and have been shown to suppress a variety of pathogens (Palaniyandi *et al.*, 2011). Among the different actinomycetes, *Streptomyces* spp. have the potential to control fungal pathogens of diverse plant hosts (Palaniyandi *et al*., 2011). There are numerous volatile compounds, antibiotics and lytic enzymes (Boukaew *et al.*, 2013; Kim *et al.*, 2015). *Streptomyces* spp. can produce volatile oils for suppressed the growth of four plant pathogenic fungi (*Rhizoctonia solani* PTRRC-9, *Pyricularia grisea* PTRRC-18, *Bipolaris oryzae* PTRRC-36 and *Fusarium fujikuroi* PTRRC-16) (Boukaew *et al*., 2013). Moreover, antibiotics present in *Streptomyces* are commercially used in pest control. Jinggangmycin, an antibiotic produced by *S. hygroscopicus* var. jinggangensis, is used to control *Rhizoctonia solani* caused rice sheath blight caused by in China (Kim *et al*., 2015).

The present study evaluated the efficacy of crude extract substances from *Streptomyces* spp. controlling *C. gloeosporioides* caused anthracnose of chili.

Materials and methods

Fungal pathogen

Collectotichum gloeosporioides was obtained from Department of Plant Pathology, Faculty of Agriculture at Kamphaengsaen, Kasetsart University Kamphaengsaen Campus, Thailand which had been isolated from a chili

anthracnose in Nakron Pathom province, Thailand. The fungal pathogen was grown on potato dextrose agar (PDA) at 28°C under white fluorescent light with a 12-h photoperiod for 14 days.

Antagonist strains

Bacterial antagonists-34 isolates of *Streptomyces* spp. were isolated from soil in Ratchaburi province and Surat Thani province by Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University and Department of Microbiology, Faculty of Science, Kasetsart University, Thailand. Each isolate was grown and maintained in Yeast Extract-Malt Extract Agar (YMA) at 30°C for 7 days.

Determination of antagonistic activity of Streptomyces spp. against fugal pathogen

Thirty-four antagonistic isolates were evaluated for antagonistic activity against *C. gloeosporioides* using the dual culture test. The experiments were conducted in a randomized complete block design (CRD) with five replications of each treatment. *Streptomyces s*pp. isolates were inoculated 2 cm away from the margin of a Petri dish containing PDA. After that five millimetre of diameter mycelia agar plug cut from actively growing mycelium of *C. gloeosporioides* using a cork borer was placed 2 cm away from the margin of the opposite site of the PDA and incubated at 30°C for 7 days. There were five replicate plates per treatment and per isolates of *C. gloeosporioides*. Radius growth of C. *gloeosporioides* was measured. Two different experiments were employed as the control groups: the first control group (Rpc) used *C. gloeosporioides* only, and the second control group (Rnc) used 50 µl of 0.75 mg/ml benomyl in 0.6 cm diameter paper disc instead of *Streptomyces* spp. isolates. At the same time, *Streptomyces* spp. isolates grew more than 2 cm, the radius of *C. gloeosporioides* (Rt) in experimental groups and control groups were measured using the equation: The percent inhibition of radial growth (RI) = [(Rpc-Rt)/Rpc] x100. The percent inhibition of radial growth was analysed by One-way ANOVA, followed by DMRT. A $P<0.05$ was considered to be statistically significant.

Preparation of crude extract substances from the most effective isolate

The most effective isolate was cultured in yeast extract-malt extract broth (YMB) and incubated at 30 ºC and 150 rpm shaking speed in a shaker incubator

for 14 days and then centrifuged at 5,000 rpm for 15 min. The upper aqueous phase was extracted by ethyl acetate. The solvent solution-ethyl acetate fraction was separately extracted and dried by using a rotary vacuum evaporator. Crude extract was weighed, placed in a small bottle and then stored at 4ºC for testing.

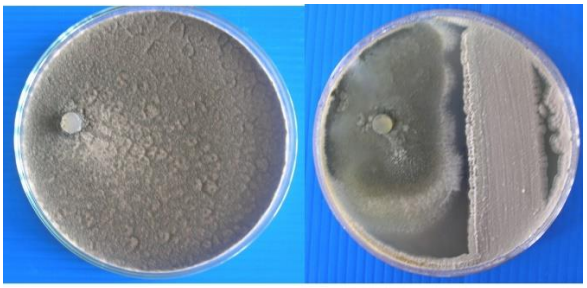
Determination of the crude extract substances from the most effective isolate in inhibiting C. gloeosporioides

The most effective isolate was extracted with ethyl acetate and tested against microbial pathogen using the disc diffusion method. The experiment was conducted using a CRD with 5 replications in each treatment. The experiment groups (treatments), was crude extract substances from the most effective isolate which was first dissolved in a small amount of dimethyl sulfoxide (DMSO) and then diluted to the desired final concentrations (87.7 mg/ml) in 5% (V/V) DMSO/water. Fifty microliter of crude extract substances in 0.6 cm diameter paper disc was placed onto 2 cm the one side of a Petri dish containing PDA medium. While, five millimetre of diameter mycelia agar plug cut from actively growing mycelium of *C. gloeosporioides* using a cork borer was placed 2 cm away from the margin of the opposite site of the PDA. The control treatments 1 and 2 were 50 µl of 0.75 mg/ml benomyl and 5% (V/V) DMSO in 0.6 cm diameter paper disc instead of crude extract substances. In addition, the control treatments 3 was 5 mm of diameter mycelia agar of *C. gloeosporioides*. The dishes were incubated at 30°C for 7 days. The percent inhibition of radial growth was calculated. The data were statistically compared using analysis of variance (One-way ANOVA). Significant differences among treatment means were determined using DMRT at $P< 0.05$.

Results

Determination of antagonistic activity of Streptomyces spp. against fugal pathogen

The dual culture assay showed that 19 isolates of antagonistic bacteria inhibited the mycelial growth of *C. gloeosporioides* (Fig 1). Thirty-four isolates of *Streptomyces* spp. were isolates of *Streptomyces* spp. presented a diverse inhibition against *C. gloeosporioides*. The percent inhibition of radial growth varied from 0.0% to 59.0%. *Streptomyces* isolate CH15 was the highest percent inhibition of radial growth of *C. gloeosporioides* and the percent inhibition of radial growth of *C. gloeosporioides* from *Streptomyces* isolate CH15 4 was not significantly different from 0.75 mg/ml benomyl.



A B

Figure 1. Antagonistic activity of *Streptomyces* isolate CH15, normal growth of *C. gloeosporioides* (A); *Streptomyces* isolate CH15 against *C. gloeosporioides* (B)

Determination of the crude extract substances from the most effective isolate in inhibiting C. gloeosporioides

The crude extract substances produced by *Streptomyces* isolate CH15 was selected for evaluation to inhibit the mycelial growth of *C. gloeosporioides* because of the highest percent inhibition of radial growth of fungal pathogen. The results showed that crude extract substances of isolate could inhibit the mycelial growth of *C. gloeosporioides*. The percent inhibition of radial growth of *C. gloeosporioides* crude extract substances from *Streptomyces* isolate CH15 was 51.2% which was not significantly different from 0.75 mg/mL benomyl in Table 1.

Table 1. The percentages of the inhibition of *C. gloeosporioides* by crude extract substances of *Streptomyces* isolate CH15 against *C. gloeosporioides* as compared with 0.75 mg/mL benomyl, same concentration used by farmers

Substances	**The percentages of the inhibition of *C. gloeosporioides* (%)**
5% (V/V) DMSO	$0.0\pm0.0^{b*}$
0.75 mg/mL benomyl	49.4 ± 0.9^{a}
87.7 mg/mL crude extract *Streptomyces* isolate CH15	51.2 ± 2.1^{a}

*Means±SD in the same column followed by a common letter were not significantly different from ^{b} ($P<0.05$).

Discussion

The potent *Streptomyces* isolates inhibited growth of *C. gloeosporioides* because *Streptomyces* spp. produced secondary metabolites or enzymes. A recent report showed that *Streptomyces* produced cell wall degrading enzymes such as chitinase and β-1, 3-glucanase (Kusama *et al*., 1986).

Moreover, antibiotics from *Streptomyces* controlled fungal pathogens (Bibb, 1996; Wang *et al*., 2015). Several researchers have already reported similar antifungal activity of *Streptomyces* against fungal pathogens. Kumar *et al.* (2014) reported *Streptomyces* sp. SCA 7 were isolated from soil samples collected from an agriculture field in India and *Streptomyces* sp. SCA 7 was assessed for antagonistic activity against two pathogenic fungi. In addition, Nguyen *et al.* (2015) exhibited that antifungal metabolites from *Streptomyces griseus* H7602 were could inhibit mycelial growth of *Phytophthora capsici* in vitro assays.

However, Thirty-four isolates of *Streptomyces* spp. showed varying degree of antifungal activity. These results confirm that isolates of *Streptomyces* are able to produce a wide variety of antifungal activity. similar results Fguira *et al.* (2012) studied 68 *Streptomyces* strains isolated from the soil sample collected from Tunisian oases ecosystem. The results showed that *Streptomyces* strains had their capacity to a broad-spectrum activity against fungal pathogens.

The ethyl acetate *Streptomyces* isolate CH15 could inhibited the mycelia of *C. gloeosporioides*; therefore, the extracellular products of *Streptomyces* isolate CH15 was important substances for controlling fungal pathogen. Islam *et al*. (2009) applied that the extracellular products of *Streptomyces* albidoflavus C247C247 showed potent antimicrobial activity against various pathogenic fungi and bacteria.

The *Streptomyces* isolate CH15 was extracted with ethyl acetate; therefore, the crude extract substances of isolate could inhibit the mycelial growth of *C. gloeosporioides*. Ethyl acetate was used extensively to extract secondary metabolites. Ghadin *et al*. (2008) reported that the extraction of secondary metabolites by ethyl acetate extract showed good activity against *C. gloeosporioides*. Similar results were reported by Khattab *et al*. (2016) who found that the ethyl acetate crude extract of two selected strains *Streptomyces* (SP 1 and SP 28) isolates were exhibited high activity against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. In contrast study by Kumara *et al*. (2014) showed that methanol extract of *Streptomyces* strain SCA 7 showed good activity against five Gram-positive and seven Gram-negative bacteria and two pathogenic fungi.

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