CHAPTER 5

Effects of the Antifungal Metabolites Isolated from Culture Filtrates of *Streptomyces rochei* ERY1 and *Streptomyces albus* subsp. *albus* PRE5 on Damping-off Control

5.1 Introduction

The infestations of soil-borne pathogenic fungi have been demonstrated a very serious problem for crop productions especially at the seedling stage. Damping-off pathogenic fungi such as *P. ahanidermatum*, *R. solani* and *S. rolfsii* can produce propagules that could survive in fluctuation condition, high temperature soil, dry soil or soilless media, for years (Hasegawa *et. al*., 2006). Wide host range (over 200 genera vegetable species) of pathogens resulted in crop yield loss throughout tropical, sub-tropical and other warm temperate regions (Edmunds and Gleason, 2003; Yaqub and Shahzad, 2005). Controlling of these pathogens, widely used and long term used of fungicide would have negative effects upon ecological system. Therefore, natural control has been considered by many researchers as an alternative plant disease control (Punja 1985; Strobel *et al*., 2004; Fawzi *et al*., 2009; Qin *et al*., 2011).

According to Chapter 4, the effective strains ERY1 and PRE5 were identified as *Streptomyces rochei* ERY1 and *Streptomyces albus* subsp. *albus* PRE5, respectively. In this Chapter 5, the antifungal metabolites produced by *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 were extracted and determined the activity on damping-off disease control.

The objectives of this chapter were as follows:

1. To extract antifungal metabolites from culture filtrates of *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5
2. To determine the minimum inhibitory concentrations (MIC) of antifungal metabolites produced by *S. rochei* ERY1 and *S. albus* subsp. *albus* PRE5 for controlling *P. aphanidermatum* and *S. rolfsii*
3. To evaluate the effects of antifungal metabolites at MIC values for controlling damping-off disease

5.2 Materials and Methods

5.2.1 Extraction of antifungal metabolites from culture filtrates

A pure colony of *S. rochei* ERY1 and/or *S. albus* subsp. *albus* PRE5 was cultured on IMA-2 medium in Petri dishes and incubated in the dark at 30ºC for 7 days. Culture dishes of the strains were then transferred to ISP2 broth medium (100 ml) in 250 ml Erlenmeyer flask and incubated using horizontal shaking at 125 rpm, 30ºC for 7 days. The culture medium was then filtered through a sheet of filter paper using vacuum pump. The supernatant was collected and adjusted to pH 7 with 1 M phosphate buffer. The cultured filtrate was partitioned three times with an equal volume of ethyl acetate. The ethyl acetate fraction was collected and evaporated to dryness using rotary evaporator at 40ºC. The crude extract was kept at -20ºC until the further trials.

5.2.2 Determination of minimum inhibitory concentration (MIC) of antifungal metabolites

MIC values of the crude extracts from *S. rochei* ERY1 and/or *S. albus* subsp. *albus* PRE5 were determined using soaking method. The crude extract of each strain was dissolved in sterile distilled water to prepare the final assay concentrations at 1, 2, 3, 4, 5, 6, 8, 16, 32 and 64 mg/ml. Then, the culture disc (2 mm in diameter) of the pathogenic fungi, *P. aphanidermatum* and *S. rolfsii*, was soaked in 20 µl of the test solutions for 5 min and transferred to culture on PDA medium plate in the dark at 25ºC. Fungal colony diameter was measured after incubation for 18, 24, 43, 64 and 112 h. The inhibition percentage was calculated by reference to the growth of the untreated control using the following formula:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Colony diameter in treatment}}{\text{Colony diameter in control}}\right) \times 100$$

The minimum concentration required for 90% (MIC_{90}) growth inhibition were calculated from the regression equation of the concentration response curves using GraphPad Prism.

5.2.3 Effects of Antifungal metabolites of the strains on the control of damping-off of Chinese cabbage seedling

1) *Streptomyces rochei* ERY1 on the control of damping-off caused by *Pythium aphanidermatum*

Chinese cabbage seeds were surface sterilized by soaking in 1% sodium hypochlorite for 1 minute, dried on sterilized tissue paper and planted in sterilized soil in plastic plug tray (5×5 cm). After planting for 15 days, twenty seedlings were transferred to the soil inoculated with *P. aphanidermatum.* The seedlings were immediately added 5 ml of the crude ethyl acetate extract of *S. rochei* ERY1 at concentrations of 4 (MIC_{90} value), 5 and 6 mg/ml, and were added again at day 4 of transplanting. Sterile distilled water was used as control treatment. The infected seedlings were counted after incubated for 7 days using disease incident. The experiment was conducted with 20 replicates and repeated twice (n = 40) for each determination using a completely randomized design (CRD).

2) *Streptomyces albus* subsp. *albus* PRE5 on the control of damping-off caused by *Sclerotium rolfsii*

Chinese cabbage seeds were surface sterilized and planted as described above. After planting for 15 days, twenty seedlings were transferred to the soil inoculated with *S. rolfsii*. The seedlings were immediately added 5 ml of the crude ethyl acetate extract of *S. albus subsp. albus PRE5* at concentrations of 5 (MIC_{90} value), 6 and 7 mg/ml, and were added again at day 4 of transplanting. Sterilized water was used as control treatment. The infected seedlings were counted after incubated for 7 days using disease incident. The experiment was conducted with 20 replicates and repeated twice (n = 40) for each determination.

5.3 Results

5.3.1 MIC values of the antifungal metabolites

1) *Streptomyces rochei* ERY1

Crude extract obtained from culture filtrates of *S. rochei* ERY1 at the concentrations 1, 2, 3, 4, 5, 6, 8, 16, 32, 64 mg/ml showed inhibitory activities on the growth of *P. aphanidermatum* (Figure 5.1). The extract at all concentrations completely inhibited mycelial growth of the fungus after incubation for 18 h and 24 h. *P. aphanidermatum* showed a slightly growth when treated with crude extract at 1 and 2 mg/ml after incubation for 48 h and grew fully the Petri discs after incubation for 63 h and 112 h. Interestingly, no colony growth was observed for treatments with crude extract at 3 – 64 mg/ml during 112 h and similar results were observed up to 15 day of incubation, suggesting that the crude extract destroyed mycelia of the fungus. The concentration of *S. rochei* ERY1 crude extract responded to inhibited *P. aphanidermatum* at 90% (MIC_{90}) value was calculated to be 3.58 mg/ml.

2) *Streptomyces albus* subsp. *albus* PRE5

Crude extract obtained from culture filtrates of *S. albus* subsp. *albus* PRE5 at the concentrations 1, 2, 3, 4, 5, 6, 8, 16, 32, 64 mg/ml showed inhibitory activities on the growth of studied *S. rolfsii* (Figure 5.2). The inhibitory activity increased with increasing concentrations of the crude extract. The extract at 3 – 64 mg/ml completely inhibited mycelial growth of the fungus after incubation for 18 h, but the growth increased and reached control growth after incubation for 112 h. The MIC_{90} value of *S. albus* subsp. *albus* PRE5 crude extract on the growth of *S. rolfsii* was 5.19 mg/ml. These results suggest that the extract could not destroy *S. rolfsii* mycelia, but delay the growth and development.

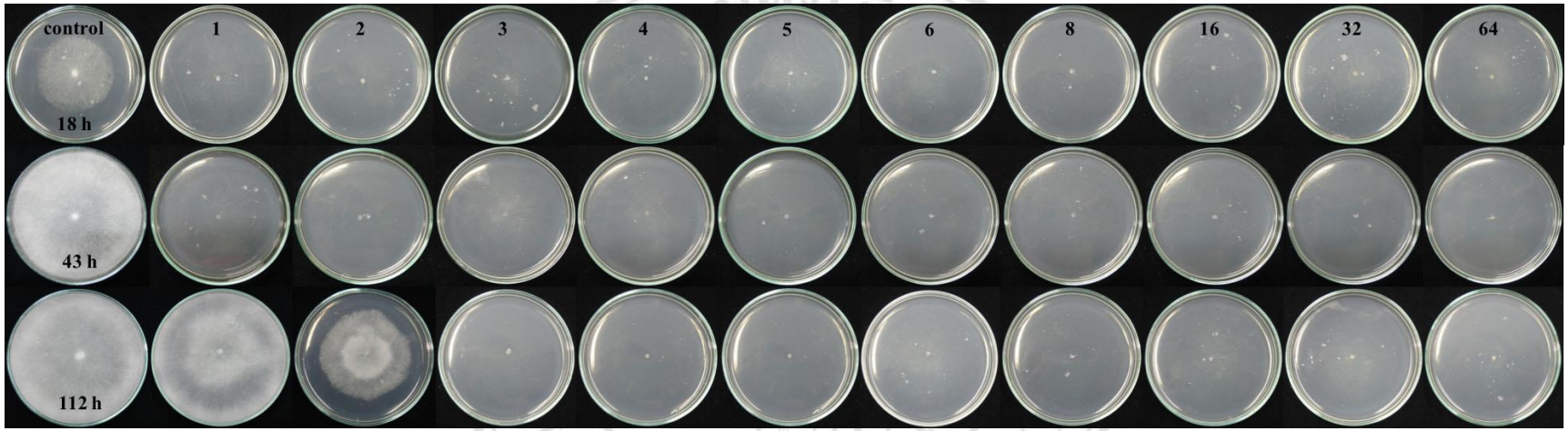

Figure 5.1 Inhibitory activities of crude extract of *Streptomyces rochei* ERY1 at concentrations 1, 2, 3, 4, 5, 6, 8, 12, 32, and 64 mg/ml on the growth of *Pythium aphanidermatum* compared with control treatment, after incubation for 18, 43 and 112 h

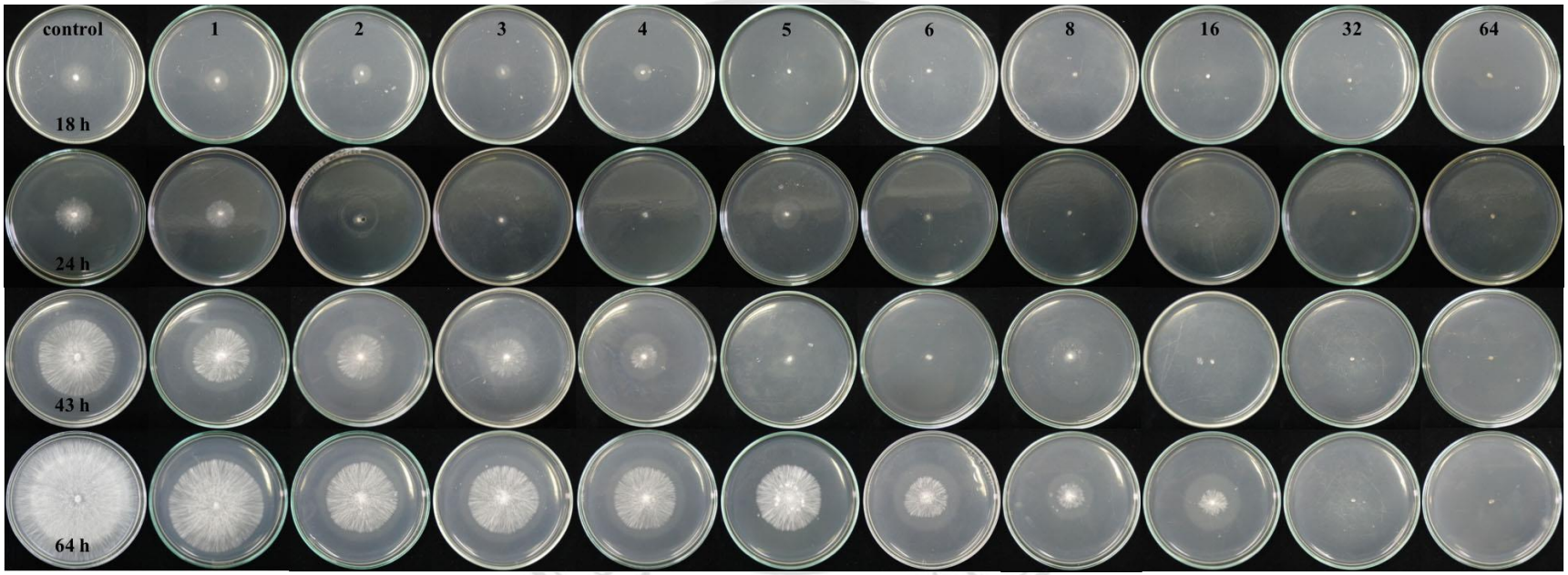

Figure 5.2 Inhibitory activities of crude extract of *Streptomyces albus* subsp. *albus* PRE5 at concentrations 1, 2, 3, 4, 5, 6, 8, 12, 32, and 64 mg/ml on the growth of *Sclerotium rolfsii* compared with control treatment, after incubation for 18, 24, 43 and 64 h

5.3.2 Effects of Antifungal metabolites on the control of damping-off of Chinese cabbage seedling

1) *Streptomyces rochei* ERY1 on the control of damping-off caused by *Pythium aphanidermatum*

After incubation for 7 days, damping-off disease were recorded using disease incidence (Figure 5.3). Seedlings that inoculated with *P. aphanidermatum* of control treatment showed water-soaked lesions on base of stems by 45%, the seedlings collapsed and died after 10 days of incubation. Other seedlings showed dwarf symptom and some of them slowly died after 10 days of incubation. In case of seedlings treated with the crude extract of antifungal metabolites of *S. rochei* ERY1 at 4, 5 and 6 mg/ml, the seedlings showed the percentage of water-soaked lesions on base of stems by 27%, 27% and 10%, respectively. While other seedlings in the treatments were stunted. Although the treated seedlings showed dwarf symptom, it could tolerant to the infection of *P. aphanidermatum.* These results suggest that the crude extract obtained from culture filtrates of *S. rochei* ERY1 may suppress and/or inhibit mycelial growth of the fungus.

Figure 5.3 Effects of antifungal metabolites obtained from culture filtrates of *Streptomyces rochei* ERY1 at 4, 5 and 6 mg/ml on the control of damping-off caused by *Pythium aphanidermatum*, after incubation for 7 days
Control (+): uninoculated control
Control (–): incoculated control

2) *Streptomyces albus* subsp. *albus* PRE5 on the control of damping-off caused by *Sclerotium rolfsii*

Damping-off disease caused by *S. rolfsii* was recorded using disease incidence. In this experiment, the MIC_{90} value of the antifungal metabolites inhibiting mycelial growth of *S. rolfsii* was 5.19 mg/ml. The crude extract of antifungal metabolites at 5, 6 and 7 mg/ml was, however, phytotoxic to Chinese cabbage seedlings. Thus, we reduced the concentration of the antifungal metabolites to 1, 2 and 3 mg/ml for testing damping-off disease control. The results showed that all control seedlings that inoculated with *S. rolfsii* were infected by the fungus and all seedlings collapsed and died (Figure 5.4). Moreover, the fungus formed a compact of mycelium to produce its propagules, sclerotia, for the infection. In contrast, the seedlings treated with the crude extract of antifungal metabolites of *S. albus* subsp. *albus* PRE5 at 1, 2 and 3 mg/ml were infected by the fungus at 80%, 30% and 80%, respectively. In this study, the most effective concentration of the crude extract was at 2 mg/ml, suggesting that the extract at 3 mg/ml may be one of factors that caused a susceptible of seedling growth leading to the infection of *S. rolfsii.*

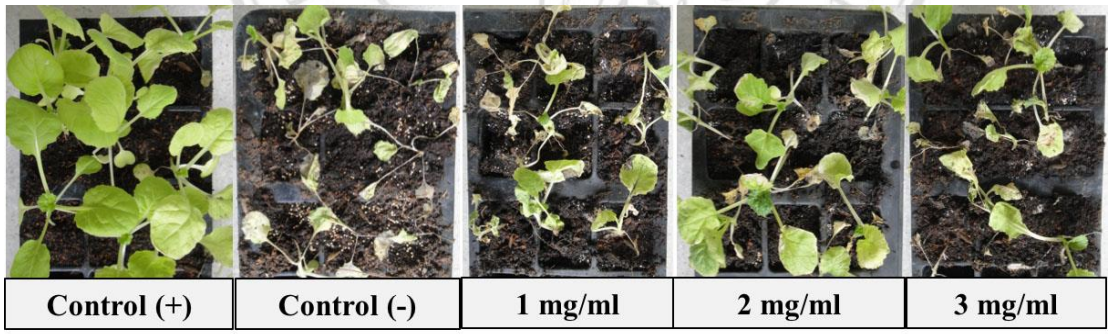

Figure 5.4 Effects of antifungal metabolites obtained from culture filtrates of *Streptomyces albus* subsp. *albus* PRE5 at 5, 6 and 7 mg/ml on the control of damping-off caused by *Sclerotium rolfsii*, after incubation for 7 days
Control (+): uninoculated control
Control (–): incoculated control

5.4 Discussion

Crude extracts of antifungal metabolites produced by *S. rochei* ERY1 and *S. albus subsp. albus* PRE5 showed inhibitory activities on the growth of damping-off pathogenic fungi, *P. aphanidermatum* and *S. rolfsii*. The effects of antifungal metabolites on the growth of pathogenic fungi were stable and/or little reduced after incubation for 15 days. It is possible that the antifungal metabolites produced by both strains could active at room temperature at least 15 days.

S. rochei ERY1 is likely that its antifungal metabolites at concentrations greater than 4 mg/ml could completely inhibited the growth and development of *P. aphanidermatum*. It is possible that the antifungal metabolites could destroy mycelial growth of the treated fungus which lead to the reduction of damping-off disease. The metabolites produced by *S. rochei*, moreover, have been reported to reduce root knot nematode disease in tomato (Ma *et al*., 2017), decrease the amount of *Fusarium solani* and *Nectria haematococca* pathogens in rhizosphere of *Amorphophallus konjac* (He *et al.*, 2015). Pazhanimurugan *et al*. (2016) reported the bioactive compounds produced by *S. rochei* were in terpenoid class compounds.

However, the antifungal metabolites produced by *S. albus* subsp. *albus* PRE5 showed phytotoxic to Chinese cabbage seedlings at concentrations at 5 mg/ml and greater than. It is possible that compounds in the crude extract affected not only mycelial growth of *S. rolfsii* (*in vitro*) but also seedling stage of Chinese cabbage (*in vivo*). However, antifungal metabolites at 2 mg/ml, the concentration lower than MIC_{90} value, could suppress the infection of *S. rolfsii* or induce the tolerant damping-off disease in Chinese cabbage seedlings. Although a relevant study about the antimicrobial activity of *S. albus* subsp. *albus* is very few and there is no report on its metabolites against plant disease, *S. albus* subsp. *albus*, in this study, has effective antifungal metabolites that could be a potential candidate for develop bio-products of damping-off disease control.

5.5 Conclusion

The antifungal metabolites produced by *S. rochei* ERY1 and *S. albus subsp. albus* PRE5 had inhibitory activities on mycelial growth of *P. aphanidermatum* and *S. rolfsii*, with MIC_{90} values at 3.58 and 5.19 mg/ml, respectively. The activities were stable or slightly decreased after 15 days. The antifungal metabolites of both strains could, moreover, control damping-off of Chinese cabbage seedlings.