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Efficacy of Crude Extract of Antifungal Compounds Produced from *Bacillus subtilis* on Prevention of Anthracnose Disease in *Dendrobium* Orchid

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Abstract

The aim of this study was to evaluate the antifungal efficacy of crude extracts of antifungal compounds produced from *Bacillus subtilis* SSE4 against plant fungal pathogen; *Colletotrichum gloeosporioides*. Antifungal compounds in culture filtrate were extracted by ethyl acetate, hexane or dichloromethane and assessed for their efficacy to inhibit the growth of *C. gloeosporioides* on agar plates and for prevention of anthracnose disease in *Dendrobium*. The results showed that crude extracts of antifungal compounds extracted by all solvents were able to inhibit the growth of *C. gloeosporioides*. However, antifungal compounds extracted by ethyl acetate and hexane showed higher percentages of fungal growth inhibition than that of antifungal compounds extracted by dichloromethane. In addition, the application of crude extract of antifungal compounds extracted by ethyl acetate showed higher efficacy on prevention of anthracnose development in orchid leaves than that of crude hexane extract of antifungal compounds. The efficacy of crude ethyl acetate extracts of antifungal compounds on anthracnose prevention was similar to that of chemical fungicide application. These results indicated that antifungal compounds extracted by ethyl acetate as biofungicide could replace chemical fungicides for orchid cultivation.

Keywords: antifungal compounds; *Bacillus subtilis*; *Colletotrichum gloeosporioides*; Anthracnose; *Dendrobium*

1. Introduction

Dendrobium is an important economic plant and the most popular and highly valued orchid in the market. However, orchid cultivation has faced several problems, particularly fungal diseases. *Colletotrichum gloeosporioides* causes anthracnose disease in a wide variety of agricultural crops including orchids (Gamagae *et al*., 2003; Prapagdee *et al*., 2008). Chemical fungicides have commonly been used for the control of phytopathogenic fungi. However, chemical fungicides lack specificity for phytopathogenic fungi resulting in an increase of fungicide resistance in phytopathogenic fungi. This problem leads to overuse of chemical fungicides. Fungicides applied in the fields are washed and leached into surface water and groundwater and soil. Fungicides contaminating the environment can enter the human body via the food chain and may cause adverse effects on human health.

With increasing environmental contamination with chemical fungicides, environmental friendly methods for control of pathogenic fungi are urgently needed to cope with these problems. Recent successes in biological control of plant diseases indicate that microbial antagonists are able to reduce populations of pathogens. Microbial antagonists are the most widely used microbes against plant pathogens (Uddin and Viji, 2002). Antagonistic organisms, particularly bacteria, 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. The mechanisms of bacteria able to reduce fungal plant diseases have also been investigated, including competition for nutrients, production of antibiotics, and secretion of lytic enzymes, as well as inducing induction of resistance systems in the host plant (Chernin and Chet, 2002; Hajek, 2004). *B. subtilis* has been observed to inhibit *Colletotrichum* spp. *in vitro* and *in vivo* (Douville and Boland, 1992; Kelemu and Badel, 1994). In addition, *B. subtilis* has been used successfully to control a diverse selection of plant pathogenic fungi and bacteria including *Botrytis cinerea, Fusarium graminearum, Sclerotium sclerotiorum, Xanthomonas oryzae* and *Pseudomonas solanacearum* (Lin *et al*., 2001; Chan *et al*., 2003; Souto *et al*., 2004; Touré *et al*., 2004).

Our previous study reported that extracellular antifungal compounds secreted into the culture filtrate of *B. subtilis* SSE4 during stationary phase of growth strongly inhibited the growth of *C. gloeosporioides* and protected the orchid from anthracnose disease (Thasana *et al*., 2010). This research focused on the antifungal

potential against the growth of *C. gloeosporioides* of crude extracts of extracellular antifungal compounds produced by *B. subtilis* SSE4. The efficacy of crude extracts of extracellular antifungal compounds on the prevention of anthracnose in *Dendrobium* was investigated.

2. Materials and Methods

2.1. Microorganisms and plant

Bacillus subtilis SSE4, bacterial antagonist, was isolated from shrimp shell waste (Thasana *et al*., 2010). It was cultured in Nutrient agar (NA). The fungal pathogen *Colletotrichum gloeosporioides* isolated from *Vanilla chamissonis* was obtained from the Faculty of Agricultural Technology, King Mongkut's Institute of Technology, Ladkrabang, Thailand. Tested fungus was grown on potato dextrose agar (PDA). Healthy, 6 month-old *Dendrobium* orchid (Eia-sakul) was purchased from an orchid garden, Thailand.

2.2. Preparation of crude extract of extracellular antifungal compounds

Bacterial cells were cultivated in Nutrient broth (NB) and incubated with continuous shaking at 28°C. The supernatant was collected at 24 hr (stationary phase) by centrifugation at 8,000 rpm for 20 min. Antifungal compounds in supernatant or culture broth were extracted by adding the equal volume of each solvent namely ethyl acetate, hexane and dichloromethane and shaken vigorously for 1 hr. Culture broth was extracted twice with each solvent for complete extraction. The solvent fractions that contained antifungal compounds were combined and concentrated by evaporation. The concentrated crude extract of the extracellular antifungal compounds was then dissolved in dimethyl sulfoxide for further use in the *in vitro* antifungal activity assay.

2.3. In vitro antifungal activity of crude extract of antifungal compounds

Each crude extract of antifungal compounds extracted by ethyl acetate, hexane or dichloromethane was added to warm molten PDA (at 45°C) to yield a final concentration of 20, 40 and 80 µg/ml, respectively. An equal volume of sterile distilled water was added to the control plate instead of the crude extract of antifungal compounds. PDA plates were seeded with 6-mm-diameter mycelial plugs of a 4-day-old of *C. gloeosporioides* in the center of the PDA plate and incubated at 28°C in the dark. Mycelial growth was measured until the fungal mycelia on the control plate reached the edge of the plate. Fungal growth inhibition was expressed as the percentage of radial growth inhibition relative to the control.

2.4. In vivo bioassay of the efficacy of crude extracts of antifungal compounds on protecting orchid from anthracnose disease

In vivo assay for prevention of the anthracnose caused by *C. gloeosporioides* was performed with 6-month-old *Dendrobium* orchids cultivated by the Good Agriculture Practice method. The experiment used a completely randomized design which was divided into 6 treatments with 10 replications. Crude extracts of antifungal compounds and spores of *C. gloeosporioides* were not applied in T1 as negative control. Orchid leaves were inoculated with 100 µl of spore suspension (10^4spores/ml) of *C. gloeosporioides* as positive control (T2). The surface of the orchid leaves was sprayed with 200 µl of 1 mg/ml of mancozeb as chemical antifungal agent and 200 µl of overnight cells of *B. subtilis* SSE4 for T3 and T4, respectively. T5 and T6 were sprayed with 200 µl of the 80 µg/ml of crude extracts of antifungal compounds extracted with ethyl acetate and hexane on the abaxial surface of orchid leaf, respectively. The abaxial surface of orchid leaves was scratched with a sterile cock borer to make a wound. Then, 100 µl of spore suspension of *C. gloeosporioides* was placed on the wound in all treatments, except T1. All inoculated orchid plants were covered with water-sprayed polyethylene bags for 24 hr. The size of visible lesions on the orchid leaves were measured daily for 7 days. Disease severity was divided into 6 categories as follows: 1) healthy, no disease symptoms of the leaves; 2) diameter of wound 0.1-0.8 cm, green leaves; 3) diameter of wound 0.8- 3.6 cm, green leaves; 4) diameter of wound 3.6-5.5 cm, rather yellow leaves; 5) diameter of wound 5.5-7.3 cm, yellow leaves and 6) diameter of wound more than 7.3 cm or leaves dropping. The disease severity index (DSI) was calculated from all plants as following equation (Basu, 1988).

$$DSI = \frac{\Sigma \text{ (Number of plants X Category value)}}{\text{Total number of plants}}$$

3. Results

3.1. Antifungal inhibitory effect of crude extract of antifungal compounds on the growth of C. gloeosporioides

According to the *in vitro* antifungal assay, crude extracts of the antifungal compounds extracted by ethyl acetate, hexane and dichloromethane showed

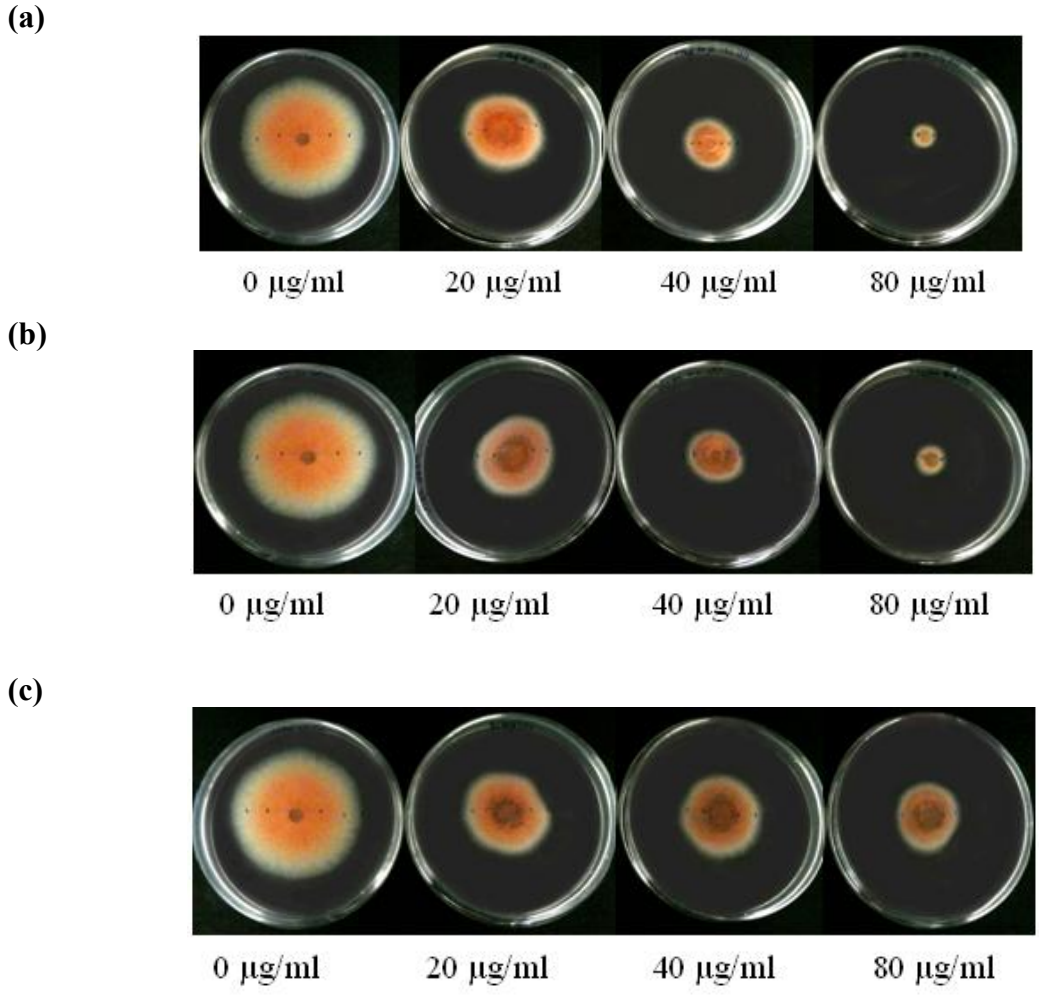

Figure 1. Antifungal activity of antifungal compounds produced from *B. subtilis* SSE4 against *C. gloeosporioides* on PDA plates amended with different concentrations of antifungal compounds extracted by (a) ethyl acetate, (b) hexane and (c) dichloromethane.

fungal growth inhibition on PDA plates supplemented with crude extracts of antifungal compounds (Fig. 1). These results indicated that fungal growth suppression resulted from the presence of extracellular antifungal compounds in the stationary culture filtrate of *B. subtilis* SSE4. The percentages of radial growth inhibition by crude extracts of antifungal compounds extracted by ethyl acetate, hexane and dichloromethane are shown in Figs. 2(a-c), respectively. Crude extracts of antifungal compounds extracted by ethyl acetate and hexane were more effective in inhibiting the fungal growth than that of dichloromethane extraction. The highest percentages of fungal growth inhibition were observed with ethyl acetate and hexane extracts after 2 days of incubation by 76.2 ±5.2 % and 75.3 ± 6.5%, respectively.

However, the growth inhibitory effects of culture filtrates decreased with the extension of fungal incubation period. Antifungal activity of crude extract of antifungal compounds increased when concentration of crude extract increased, and vice versa. Owing to the lower antifungal activity of dichloromethane extracts ethyl acetate and hexane extracts were selected for *in vivo* bioassay.

3.2. Prevention of anthracnose disease on Dendrobium leaves by crude extract of antifungal compounds

To evaluate the potential of crude ethyl acetate and hexane extracts of antifungal compounds on protecting *Dendrobium* leaves from *C. gloeosporioides* infection, bioassays were performed to measure the ability crude extracts of antifungal compounds of *B. subtilis* SSE4 to suppress fungal infection. The results of *in vivo* biocontrol for suppression of anthracnose disease by crude extracts of antifungal compounds extracted by ethyl acetate and hexane (T5 and T6) and overnight cells of *B. subtilis* SSE4 (T4) showed that the disease severity index (DSI) was significant reduced ($p<0.05$) compared to the positive control treatment (T2) (Table 1).

Fig. 3 demonstrates the anthracnose lesion on orchid leaves. The visible foliar symptom of anthracnose disease appeared 2 days after fungal inoculation in the

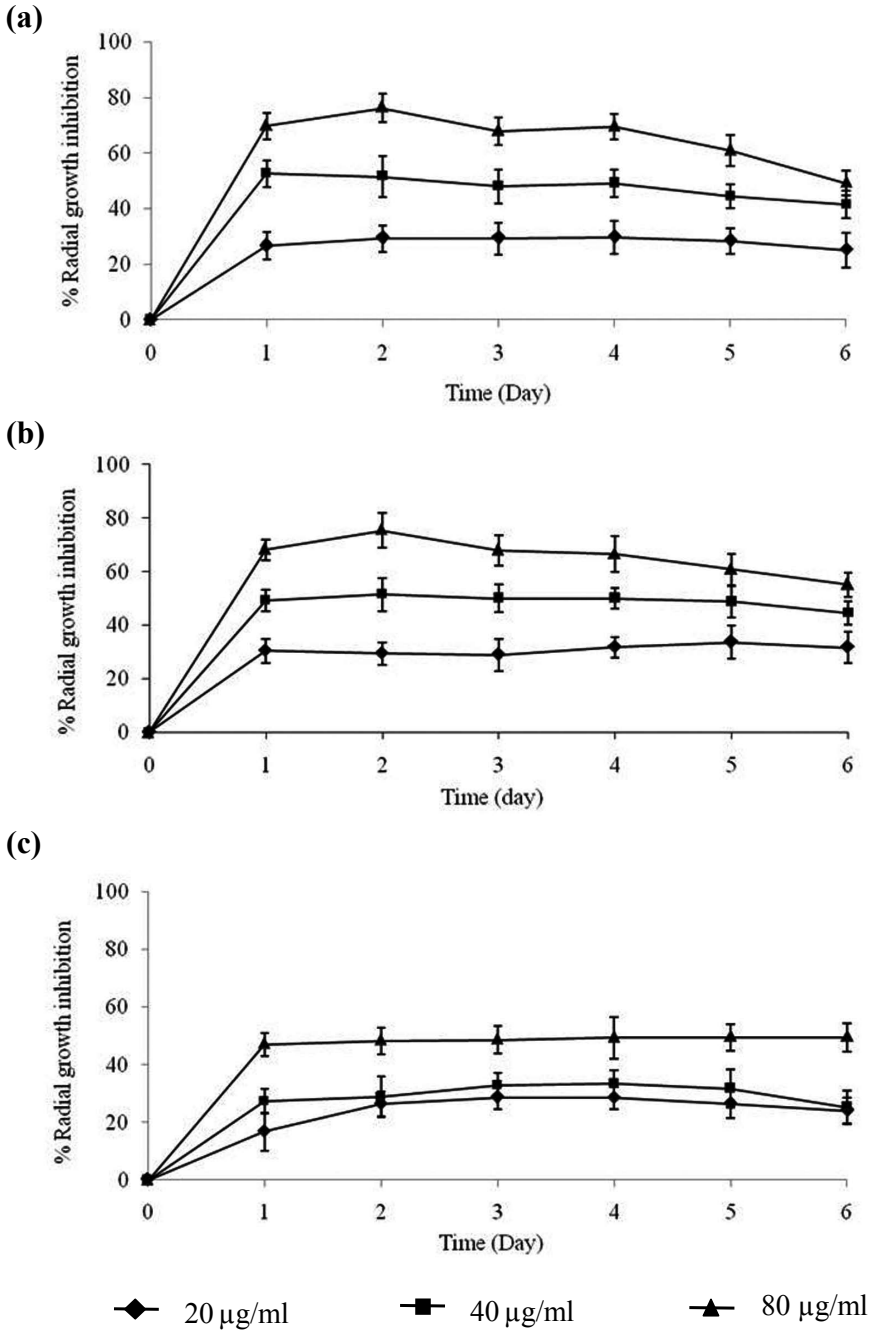

Figure 2. Antifungal activity of various concentrations of antifungal compounds extracted by (a) ethyl acetate, (b) hexane and (c) dichloromethane of *B. subtilis* SSE4 as observed by the growth inhibition of *C. gloeosporioides*. (means ± SEM; *n=5*)

positive controls (T2). The wound spots expanded and merged to cover the whole affected area. The lesion developed daily with an increase in dead tissue until the leaves turned to yellow and finally dropped off. Complete anthracnose disease suppression occurred after application of mancozeb (T3) and crude extract of antifungal compounds extracted by ethyl acetate (T5) on orchid leaves before application of fungal spores. Disease appearances of orchid leaves in T3 and T5 were similar to that of T1 (negative control treatment). The DSI of treatments that were applied overnight cells of *B. subtilis* SSE4 (T4) and crude extract of antifungal compounds extracted by hexane (T6) were higher than that of T3 and T5 ($p<0.05$). These results indicated that overnight cells of *B. subtilis* SSE4 (T4) and crude extract of antifungal compounds extracted by hexane had lower efficacies on prevention of anthracnose than crude extract of antifungal compounds extracted by ethyl acetate.

4. Discussion

B. subtilis SSE4 isolated from shrimp shell waste is a potential bacterial antagonist. Both exponential

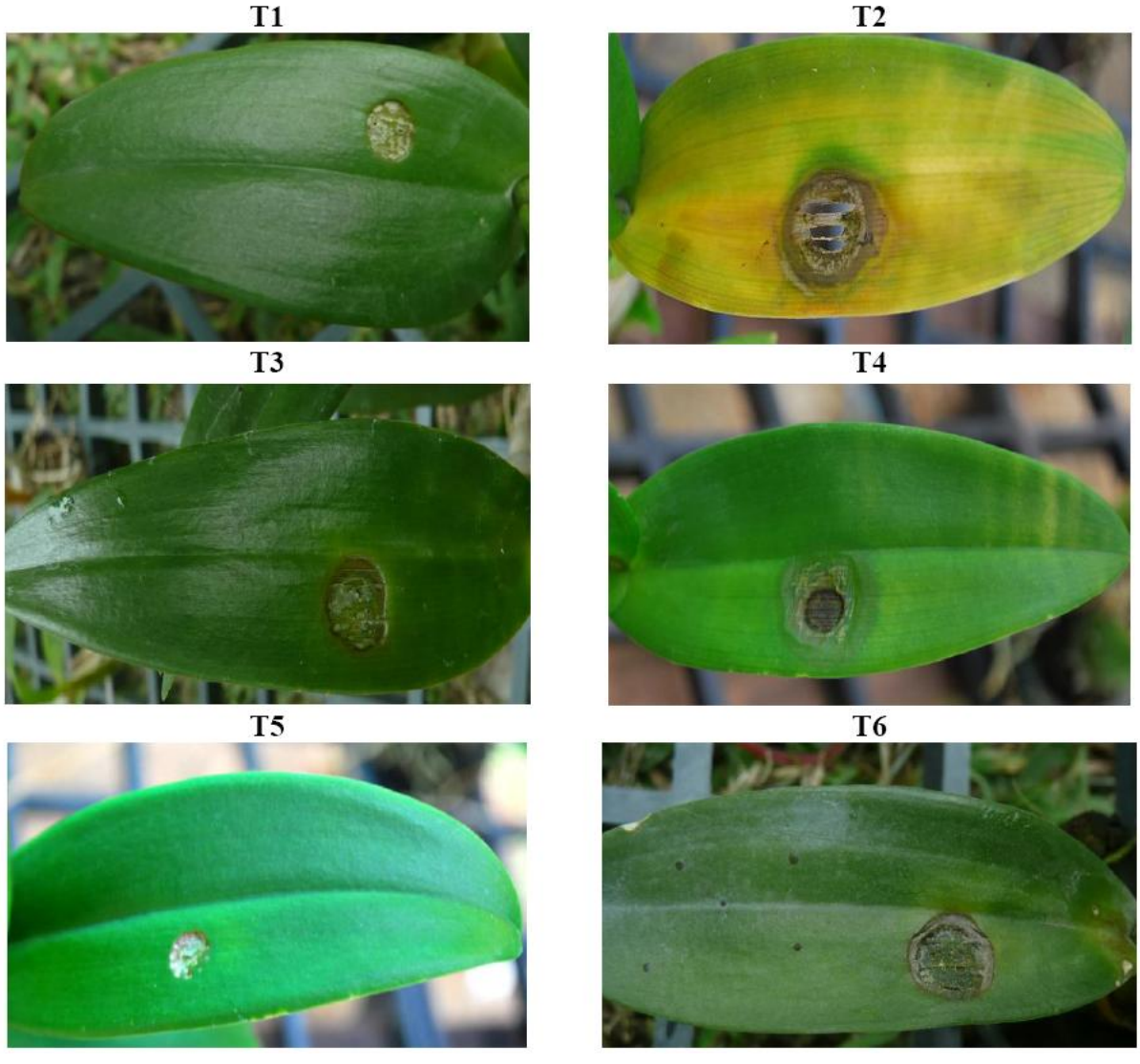

Figure 3. Visible symptoms of anthracnose disease on *Dendrobium* orchid with different applications on 7 days after infection of *C. gloeosporioides*.

T1: Negative control (No fungal infection)
T2: Positive control (Fungal infection)
T3: Mancozeb treatment + Tested fungus
T4: *B. subtilis* SSE4 cells + Tested fungus
T5: Antifungal compounds extracted by ethyl acetate + Tested fungus
T6: Antifungal compounds extracted by hexane + Tested fungus

and stationary culture filtrate inhibited the growth of phytopathogenic fungi; *C. gloeosporioides* and *Sclerotium rolfsii*. Its stationary culture filtrate had higher antifungal activity than the exponential culture filtrate (Thasana *et al*., 2010). Our results demonstrate that crude extracts of antifungal compounds in stationary culture filtrate of *B. subtilis* SSE4 were able to strongly inhibit the growth of *C. gloeosporioides*. The bioactive compounds that exhibit antifungal and antibacterial activities in the stationary culture filtrate of *B. subtilis* SSE4 were a cyclic lipopeptide such as subtulene A and iturin A (Thasana *et al*., 2010). Iturins have generally been shown to display strong antifungal toxicity against a variety of fungi but only limited antibacterial activity (Hiradate *et al*., 2002). The study of Kajimura *et al*. (1995) reported that *B. subtilis* FR-2 produced lipopeptide antibiotics and bacilopeptins as antifungal antibiotics.

However, the highest percentages of fungal growth inhibition by crude extracts of antifungal compounds extracted by ethyl acetate and hexane were found at 3 days after fungal inoculation; after that the percentages of growth inhibition decreased with time. Several investigators reported that decreases in the degree of growth inhibition were associated with the increases in the incubation period of the fungal culture (Chang

Table 1. Disease severity index (DSI) of anthracnose disease caused by *C. gloeosporioides* on *Dendrobium* leaves

Treatment	DSI of anthracnose on 7 days after fungal infection
T1 = No fungal infection (Negative control)	1
T2 = Fungal infection (Positive control)	6
T3 = Fungus + Mancozeb (Chemical) treatment	1
T4 = Fungus + Cells of *B. subtilis* SSE4	3
T5 = Fungus + Antifungal compounds extracted by ethyl acetate	1
T6 = Fungus + Antifungal compounds extracted by hexane	2

et al., 2007). In addition, crude extracts of antifungal compounds extracted by dichloromethane had a lower percentage of radial fungal growth inhibition than that of crude ethyl acetate and hexane extracts of antifungal compounds. This could indicate that the bioactive compounds, subtulene A and iturin A, in their culture filtrates were better dissolved in ethyl acetate and hexane than in dichloromethane. Antibiotics produced by bacterial antagonists have a broad spectrum activity against bacteria and fungi. Zwittermycin A produced by *Bacillus cereus* and *Bacillus thuringiensis* adversely affects the growth of a wide range of plant pathogenic fungi, particularly *Phytophthora* spp. and *Pythium* spp. (Raaijmakers *et al*., 2002).

Our previous study reported that the stationary culture filtrate of *B. subtilis* SSE4 prevented the development of anthracnose symptoms on orchid leaves similar thus resembling the effects of chemical fungicides (Thasana *et al*., 2010). Under greenhouse condition, the use of crude extracts of antifungal compounds extracted by ethyl acetate was as an effective agent to protect anthracnose development on orchid leaves, better than crude hexane extracts of antifungal compounds. Mckeen *et al*. (1986) reported that antibiotic substances produced by *B. subtilis* were able to control the development of stone fruit brown rot. Impurities in this antifungal compounds were removed by extraction with ethyl acetate and acetone. The crude extract at 1 mg/ml showed almost complete suppression of brown rot on peach fruit. *Bacillus amyloliquefaciens* RC-2 produced extracellular antifungal compounds, iturins, which were able to inhibit the development of mulberry anthracnose caused by *Colletotrichum dematium* (Hiradate *et al*., 2002). Our findings indicated that crude ethyl acetate extracts of antifungal compounds controlled anthracnose as effectively as the chemical fungicide control.

5. Conclusion

B. subtilis SSE4 produced extracellular antifungal compounds that were able to inhibit the growth of *C. gloeosporioides*. Crude ethyl acetate and hexane extracts of antifungal compounds were stronger growth inhibitors of *C. gloeosporioides* on PDA plates than that of extracted by dichloromethane. In addition, crude extracts of antifungal compounds extracted by ethyl acetate exhibited a preventive action on the anthracnose, similar to that of chemical fungicides. The crude extracts of extracellular antifungal compounds extracted by ethyl acetate could therefore be used as a biofungicide for control of anthracnose in orchids.

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