



ANTIFUNGAL ACTIVITY OF BIOACTIVE COMPOUND FROM ENDOPHYTIC FUNGI ISOLATED FROM MANGROVE LEAVES

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Abstract

One hundred and fifty endophytic fungi were isolated from healthy mangrove leaves in the eastern part of Thailand. All fungi were tested for antifungal activity against indicator fungal phytopathogens; *Alternaria brassicicola* DOAC 0436, *Colletotrichum capsici* DOAC 1511, *C. gloeosporioides* DOAC 0782, *Fusarium oxysporum* DOAC 1808 and *Pestalotiopsis* sp. DOAC 1098, by dual culture method. It was found that 23 isolates (15.3%) inhibited growth of fungal phytopathogens at moderate level or higher (at level > 30.0%). Endophytic fungus BUEN 880 exhibited the best result. Growth of all indicator fungal phytopathogens were inhibited by BUEN 880 at the level of $\geq 30.0\%$. The percentage of inhibition against indicator fungal phytopathogens were as follows; *A. brassicicola* DOAC 0436 (41.7%), *C. capsici* DOAC 1511 (30.8%), *C. gloeosporioides* DOAC 0782 (37.5%), *F. oxysporum* DOAC 1808 (40.0%) and *Pestalotiopsis* sp. DOAC 1098 (33.3%). Testing of the extract from BUEN 880 culture filtrate against fungal phytopathogens by disc diffusion method revealed that *C. capsici* DOAC 1511, *C. gloeosporioides* DOAC 0782 and *Pestalotiopsis* sp. DOAC 1098 were inhibited at moderate level with the equal distance of 4 mm.

Keywords: antifungal activity, endophytic fungi, fungal phytopathogen, mangrove leaves, bioactive compound

Introduction

Mangroves are intertidal forest wetland distributed in the tropical and subtropical regions (Silva et al. 2011). Mangrove plants have morphologically and physiologically adapt to habitats with high salinity, strong wind and high temperature (Latha and Mitra 1998; Maria et al. 2005). Mangrove fungi are the second largest ecological group of marine fungi and have been realized that they are important to mangrove adaptation. They are also suggested as promising sources for screening of new products (Latha and Mitra 1998). Endophytic fungi from mangroves colonized and grew asymptotically within healthy tissues of mangrove plants. The relationship of endophytic fungus with the host plant has been known as symbiotic and probably mutualistic (Gao et al. 2010; Nithya and Muthumary 2011). The various bioactive compounds produced by endophytic fungi possess unique structures and have high potential for exploitation in medicine, agriculture and industrial uses (Nithya and Muthumary 2011). In agriculture, application of large amount of synthetic fungicides has been considered to be one of the cheapest and most common approaches to control plant diseases (Rabea and Steurbaut 2010). These fungicides usually difficult to degrade and then cause toxicity to humans and animals. Phytopathogens also have been developed resistance to frequent pesticides-using, lead to the decreasing of efficiency (Gao et al. 2010). Therefore, the search for bioactive compounds from terrestrial and marine-derived endophytic fungi



which are safe and more environmentally friendly were introduced to replace the synthetic fungicides. The main aim of this study was to isolate endophytic fungi from mangrove leaves in the eastern part of Thailand and to determine their antifungal activity against some common fungal phytopathogens in Thailand.

Methodology

Indicator fungi

Alternaria brassicicola DOAC 0436, *Colletotrichum capsici* DOAC 1511, *C. gloeosporioides* DOAC 0782, *Fusarium oxysporum* DOAC 1808 and *Pestalotiopsis* sp. DOAC 1098 were purchased from the DOAC Culture Collection Centre, Plant Pathology and Microbiology Division, Department of Agriculture, Thailand. The fungal cultures were maintained on potato dextrose agar slant at 28°C.

Sample collection


Healthy mangrove leaves growing in The Kung Krabaen Bay Royal Development Study Center, Chanthaburi Province; The Nature Education Center for Mangrove Conservation and Ecotourism, Chonburi Province and The Bangpakong Mangrove Eco-museum in Chachoengsao Province, Thailand, were collected from July to November 2010. The total of leaf samples were collected from 13 mangrove plant species; *Bruguiera gymnorrhiza* (L.) Savigny, *Bruguiera sexangula* Poir., *Thespesia populnea* (L.) Soland. ex Correa, *Avicennia marina* (Forsk.) Vierh., *Ceriops tagal* (Perr.) C. B. Rob., *Sonneratia alba* J. Smith, *Sonneratia ovata* Back, *Xylocarpus granatum* Koen., *Xylocarpus rumphii* (Kostel.) Mabblerley, *Acrostichum aureum* L., *Rhizophora mucronata* Poir., *Rhizophora apiculata* Bl. and *Excoecaria agallocha* L.

Isolation of endophytic fungi

Mangrove leaves were cut into pieces of 5×5 mm and were surface sterilized in 70% (v/v) ethanol for 3 min and then in 0.5% (v/v) sodium hypochlorite solution for 1 min. The leaf samples were rinsed with sterilized water for three times and blotted with sterilized filter papers (Barik et al. 2010). Pieces of leaves were directly placed on Petri dish containing potato dextrose agar (PDA) (Tayung et al. 2011). The plates were incubated at 28°C for 3-7 days. The growing fungal mycelium was cut by using cork borer diameter of 0.6 cm, placed on PDA plate and incubated at 28°C for 5 days. The fungi were identified on the basis of their morphological characteristics.

Antifungal activity test by dual culture method

Endophytic fungi and fungal phytopathogens were cultured separately on PDA plate and incubated at 28°C for 5 days. The growing fungal mycelium was cut by using cork borer diameter 0.6 cm. Fungal discs of endophytic fungi and fungal phytopathogens were placed on the opposite site of the same PDA plate, at distance of 5 cm between the fungal pair. Only fungal disc of each fungal phytopathogen was placed at one site of the PDA plate for using as control. The dual cultures were incubated at 28°C for 3-5 days. The fungal growth was determined by the radiant of fungal phytopathogen and the percentage of inhibition was calculated by the following formula (Modified from Rahman et al. (2009)).


$$\text{Percentage of inhibition} = [(R1 - R2) / R1] \times 100$$

Where R1 represents radiant of colony of fungal phytopathogen in control plate and R2 is radiant of colony of fungal phytopathogen in test sample plate. The percentage of inhibition was categorized on growth inhibition category level from low to very high antifungal activity; < 30% = low antifungal activity, 30-< 50% = moderate antifungal activity, 50-< 70% = high antifungal activity, \geq 70% = very high antifungal activity (Modified from Živković et al. (2010)). The endophytic fungus with the percentage of inhibition more than 30% was kept for further study.

Fermentation and recovering of the crude extract

The fungal mycelium was cut from PDA plate by using cork borer diameter of 0.6 cm. Five fungal discs were placed into 250 ml Erlenmeyer flask containing 100 ml of potato dextrose broth (PDB). The flask was incubated at 28°C for 7 days. At the end of fermentation, the fermentation broth of endophytic fungus was separated from the mycelium by filtration through Whatman No.1 filter paper and the filtrate was extracted twice with 100 ml of ethyl acetate (1:1, v/v). The pooled extract was evaporated in a rotary vacuum evaporator at 40 ± 1 °C. The dry crude extract was then dissolved in 1ml of 50% dimethyl sulfoxide (DMSO) and was kept for antifungal activity testing.

Antifungal activity testing by disc diffusion method

Indicator fungal phytopathogen was cultured on the center of PDA plate and incubated at 28°C for 3 days. The crude extract containing discs (20 μ l) were placed on PDA plate with the distance of 1.5 cm apart from the fungal phytopathogen. The control disc was filled with 50% DMSO. The Petri dish was incubated at 28°C for 3 days. Then, the fungal growth was determined by the inhibition distance between crude extract discs and the mycelium of the fungal phytopathogen compared to the control disc. Inhibition distance (ID) of antifungal activity by disc diffusion method was classified as follow; \leq 2mm = low antifungal activity, > 2-6 mm = moderate antifungal activity, > 6-10 mm = high antifungal activity, > 10 mm = very high antifungal activity.

Results

Screening of antifungal phytopathogens by dual culture method

Among 150 endophytic fungal isolates which were tested for antifungal activity by dual culture method, 23 isolates (15.3%) inhibited growth at least one of the fungal phytopathogens at the level of more than 30%. Endophytic fungus BUEN 880 showed the best results, this fungus inhibited growth of five indicator fungal phytopathogens. The percentage inhibition activity against indicator fungal phytopathogens were as follows; *A. brassicicola* DOAC 0436 (41.7%), *C. capsici* DOAC 1511 (30.8%), *C. gloeosporioides* DOAC 0782 (37.5%), *F. oxysporum* DOAC 1808 (40.0%) and *Pestalotiopsis* sp. DOAC 1098 (33.3%) (Figure 1).

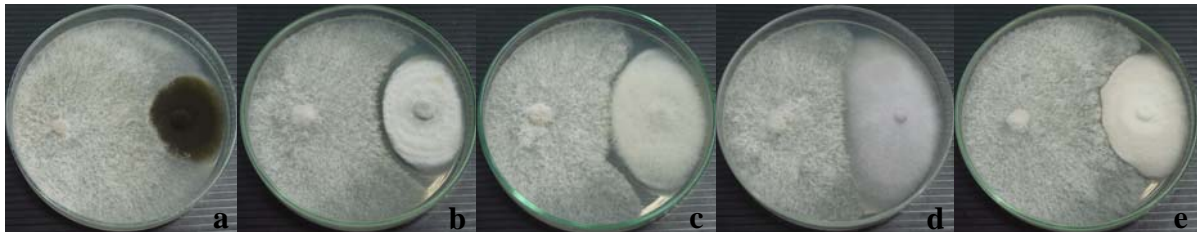


Figure 1 Antifungal activity of endophytic fungus BUEN 880 (colony on the left of plate) against fungal phytopathogens (colony on the right of plate); a: against *A. brassicicola* DOAC 0436, b: against *C. capsici* DOAC 1511, c: against *C. gloeosporioides* DOAC 0782, d: against *F. oxysporum* DOAC 1808, e: against *Pestalotiopsis* sp. DOAC 1098.

Antifungal phytopathogens by disc diffusion method

Among 23 crude extracts of endophytic fungi, only 5 extracts (21.7%) exhibited antifungal activity against indicator fungal phytopathogens by disc diffusion method. All of these extracts inhibited growth of fungal phytopathogens at moderate level (inhibition distance ranging from 2 to 6 mm). Extract of BUEN 880 showed the best result, this extract inhibited growth of fungal phytopathogens; *C. capsici* DOAC 1511, *C. gloeosporioides* DOAC 0782 and *Pestalotiopsis* sp. DOAC 1098 at the distance of 4 mm, 4 mm and 4 mm, respectively (Figure 2). The inhibition results of crude extract differ from the results of dual culture method, since the extract did not inhibited growth of *A. brassicicola* DOAC 0436 and *F. oxysporum* DOAC 1808.

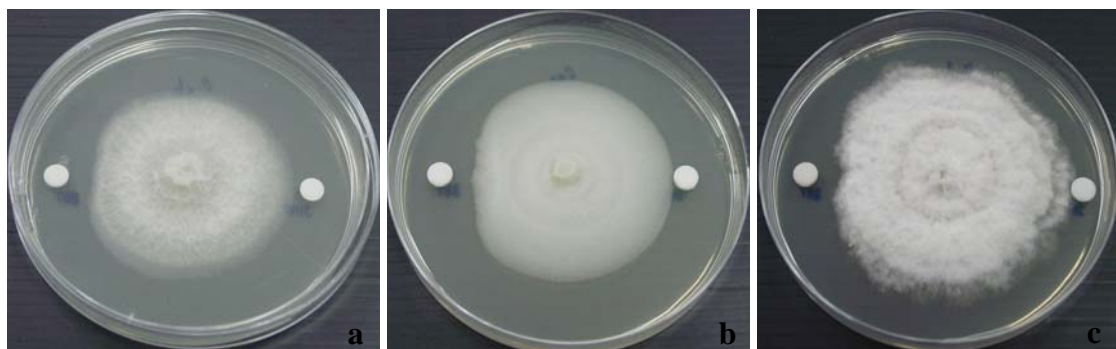


Figure 2 Antifungal activity of crude extract of BUEN 880 (the left of plate) against fungal phytopathogens (center of the plate); a: *C. gloeosporioides* DOAC 0782, b: *C. capsici* DOAC 1511, c: *Pestalotiopsis* sp. DOAC 1098. Control disc soaked with 50% DMSO was placed on the right of the plate.

Characteristic of endophytic fungus BUEN 880

BUEN 880 was isolated from leaf of *Thespesia populnea* (L.) Sol. ex Correa. BUEN 880 grows as a white cottony colony and turning with age into greenish on PDA. Under light microscope, only hyaline septate mycelium (2.5 μ m wide) was observed. The mycelium was highly branched, conidia was not produced. Thus, at present BUEN 880 can be classified in the group of mycelia sterilia (Figure 3).

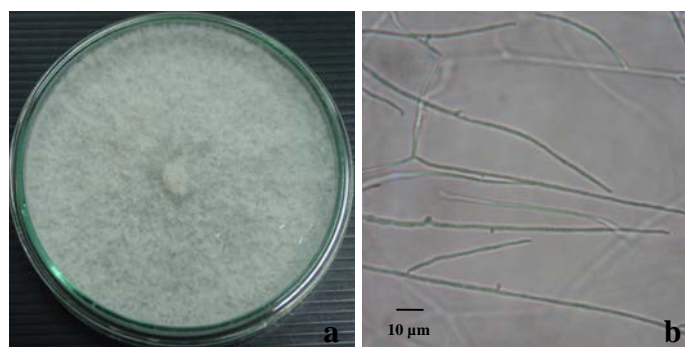



Figure 3 Characteristic of 7 days-old BUEN 880; a: colony on PDA plate, b: mycelium under a light microscope.

Discussion and Conclusion

Approximate 15.3% of the endophytic fungi isolated from healthy mangrove leaves produced secondary metabolites with antifungal activity. The percentage inhibition activity against each indicator fungal phytopathogen was different. The different values obtained when different fungal phytopathogens were used indicated that the direct interaction between endophytic fungus and pathogen was complex and may be sensitive to species-specific antagonism as previously described by Arnold et al. (2000). More than twenty one percent (21.7%) of crude extracts from fungal broth culture showed moderate inhibition activity in dual culture. BUEN 880 was the best fungus with antifungal activity. The crude extract differ from the results of dual culture method, since the extract did not inhibited growth of *A. brassicicola* DOAC 0436 and *F. oxysporum* DOAC 1808. This may be due to the fungus produce other bioactive compounds that can not be extracted. This compound may be enzyme since there has been reported that endophytes directly suppress pathogens by producing either antibiotic or lytic enzymes (Gao et al. 2010).

References

1. Arnold AE, Mejía LC, Kyllö D, Rojas EI, Maynard Z, Robbins N, Herre EA (2000) Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences of the United States of America* 100:15649-15654.
2. Barik BP, Tayung K, Jagadev PN, Dutta SK (2010) Phylogenetic placement of an endophytic fungus *Fusarium oxysporum* isolated from *Acorus calamus* rhizomes with antimicrobial activity. *European Journal of Biological Sciences* 2:8-16.
3. Gao F, Dai C, Liu X (2010). Mechanisms of fungal endophytes in plant protection against pathogens. *African Journal of Microbiology Research* 4:1346-1351.
4. Latha R, Mitra S (1998). Mangrove fungi in India. *Current Science* 86:1586-1587.
5. Maria GL, Sridhar KR, Raviraja NS (2005) Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest cost of India. *Journal of Agricultural Technology* 1:67-80.
6. Nithya K, Muthumary J (2011) Bioactive metabolite produced by *Phomopsis* sp., an endophytic fungus in *Allamanda cathartica* Linn. *Recent Research in Science and Technology* 3:44-48.
7. Rabea EI, Steurbaut W (2010) Chemically modified chitosans as antimicrobial agents against some plant pathogenic bacteria and fungi. *Plant Protection Science* 46:149-158.

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8. Rahman MA, Begum MF, Alam MF (2009) Screening of *Trichoderma* isolates as a biological control agent against *Ceratocystis paradoxa* causing pineapple disease of sugarcane. *Mycobiology* 37:277-285.
 9. Silva MRO, Almeida AC, Arruda FVF, Gusmão N (2011) Endophytic fungi from brazilian mangrove plant *Laguncularia racemosa* (L.) Gaertn. (Combretaceae): their antimicrobial potential. *Science against Microbial Pathogen: Communicating Current Research and Technological Advances* 2:1260-1266.
 10. Tayung K, Barik BP, Jha DK, Deka DC (2011) Identification and characterization of antimicrobial metabolite from an endophytic fungus, *Fusarium solani* isolated from bark of Himalayan yew. *Mycosphere* 2:203–213.
 11. Živković S, Stojanović S, Ivanović Ž, Gavrilović V, Popović T, Balaž J (2010) Screening of antagonistic activity of microorganisms against *Colletotrichum acutatum* and *Colletotrichum gloeosporioides*. *Archives of Biological Science Belgrade* 62:611-623.