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Antimicrobial activity from endophytic fungi isolated from plant leaves in Dipterocarpous forest at Viengsa district Nan province, Thailand

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Eleven fungal endophytes representing different morphotaxa were characterized from 68 cultures, which were isolated from 4 species of Dipterocapous trees (*Dipterocarpus tuberculatus* Roxb., *Shorea obtusa* Wall., *Shorea siamensis* Miq. and *Dalbergia oliveri* Gamble.) growing in the Dipterocapous forest at Viengsa district, Nan province. Species of *Phyllosticta* spp. (15 isolates), *Nodulisporium* spp. (13 isolates) and *Xylaria* sp.1 (10 isolates) were the most frequently found. All endophytic fungal isolates were tested for potential production of bioactive metabolites. They were tested for antimicrobial activity against pathogenic microorganisms such as *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aerogenosa*, *Escherichia coli* and *Candida albicans* by the paper disk susceptibility test. They inhibited the growth of Gram positive bacteria more than Gram negative bacteria. *Candida albicans* was inhibited only by *Nodulissporium* sp. (DT6) and *Xylaria* sp.1 (DO9).

Key words: endophytic fungi, antimicrobial activity, Dipterocarpous forest

Introduction

Micobial endophytes are microoganisms which grow intercellularly and asymtomatically within living tissues establishing mutual relationship with the host plants (Petrini, 1991). Fungal endophytes have also been recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities (Bills and Polishook, 1991).There are many reports about antimicrobial compounds produced by endophytic fungi in culture that are active against plant and human pathogenic microorganisms. Chareprasert *et al*.

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(2006) reported on antimicrobial activity exhibited by endophytic fungi isolated from teak and rain tree and they found to produce some metabolites active against bacteria and yeast. New bioactive metabolites (e.g. asperfumin) produced by *Aspergillus fumigatus* CY018, an endophytic fungus, has been shown to inhabit *Candida albicans* (Liu *et al*., 2004).

Fungal endophytes are considered to be a potential source for novel bioactive products (Strobel, 2003).The aim of the current research was to investigate the antimicrobial potential of endophytic fungi and this is the first report on screening of endophytic fungi from Nan province, Thailand.

Material and methods

Plant sample collection

Mature healthy plant leaves were collected by sampling from different parts of the trees (Table 1) growing in the Dipterocarpous forest at Viengsa district, Nan province. All samples were stored in sterile polythene bags in an icebox and chilled samples (4.5 ºC) were used to isolate endophytic fungi within 48 h of collection.

Isolation and identification of endophytic fungi

Discs of leaves (0.5 cm diameter) were cut using a sterile pinch cutter. Samples were washed thoroughly in running tap water. Surface of the plant tissues was treated by following the methodology of Murali *et al*. (2007). Plant tissues were immersed in 75% ethanol for 1 min and in an aqueous solution of sodium hypochlorite (2.5% available chlorine) for 15 min, followed by washing with 70% ethanol for 5 sec. The tissues were then rinsed in sterile distilled water and allowed to surface-dry under sterile conditions. The surface-sterilized samples were placed on petri dishes containing Potato Dextrose Agar (PDA) (supplemented with streptomycin (100 µg ml^{-1}) to inhibit bacterial growth) and incubated at room temperature at around 25°C

Endophytic fungi were identified according to their macro and microscopic structures. The taxa were assigned to genera following Barnett and Hunter (1998) and Von Arx (1978).

Fermentation and extraction

Seven day-old cultures on PDA of the endophytic fungi were inoculated into Malt Extract Both (MEB) in Erlenmeyer flasks 250 ml, followed by static condition and incubated for 30 days at 25°C. The fermentation broth of each

fungal endophyte was filtered, and the filtrate was extracted three times with ethyl acetate (EtOAc) at room temperature. Evaporation of the extracted solution was done in a rotary evaporator.

Table 1. Species of host plants in Dipterocarpous forest at Viengsa district, Nan province.

NO.	Plant species	Code	Family
1	*Dipterocarpus tuberculatus* Roxb.	DT	DIPTEROCARPACEAE
2	*Shorea obtusa* Wall.	SO	DIPTEROCARPACEAE
3	*Shorea siamensis* Miq.	SS	DIPTEROCARPACEAE
4	*Dalbergia oliveri* Gamble.	DO	PAPILIONACEAE

Antimicrobial activity assay

Five reference human pathogenic microorganisms were used for the antimicrobial activity assay including two Gram positive bacteria, *Staphylococcus aureus* (STAPY), *Bacillus subtilis* (BACIL) and two Gram negative bacteria, *Pseudomonas aerogenosa* (PSEUD), *Escherichia coli* (ESCHE) and the yeast, *Candida albicans* (CAND). Antimicrobial activity was determined using the paper disk susceptibility test (Wang *et al*., 2007). A sterilized filter paper was dipped into the extracts and then placed on to the lawn of reference microorganisms. The magnitude of antimicrobial activity was assessed by the diameter of inhibition zones relative to those of positive and negative controls. Streptomycin and nystatin were co-assayed as positive controls, and 10% DMSO as a negative control.

Results and discussion

From 80 leaf segments (20 from each host species) from 4 Dipterocarpous trees belonging to 2 families (Table 1), 68 isolates were obtained, representing 11 endophytic fungi of different morphotaxa (Fig. 1). The leaves of the Dipterocarpous plants were highly colonized by *Phyllosticta* spp. with 15 isolates, *Nodulisporium* spp. 13 isolates and *Xylaria* sp.1 consisting of 10 isolates (Table 2).

Hypomycetes of the Deuteromycotina are common fungal endophytes among plants inhabiting temperate, tropical and rain forest vegetations (Bacon and White 1994). Deuteromycetous fungal isolates, as endophytes in mangrove vegetations of costal Karnataka, Picchavaran and Podicherry (India) were more prevalent than members of the Ascomycotina (Maria and Sridhar, 2003; Suryanarayanan *et al*., 1998). In this study, however, coelomycetes were found to be more numerous than those of hyphomycetes and ascomycetes. From the

results, the most the common genus was *Phyllosticta* which it certainly known as the plant pathogen but may also occur endophytically.

Crude extracts of 68 fungal isolates were tested for antimicrobial activities, 50(73.52%) of endophytic fungi produced secondary metabolites antagonistic to Gram positive bacteria and 26(38.23%) of endophytic fungi could inhibit the growth of Gram negative bacteria. Moreover, 13(19.11%) could inhibit the growth of *Candida albicans* but 18(26.47%) of endophytic fungal extracts had no effect on reference microorganisms. The results show the size of inhibition zone of different endophytic fungi from each host plant (Table 3). The crude extracts of *Nodulisporium* sp. (DT6) and *Xylaria* sp.1 (DO9) exhibited antimicrobial activity against all test microorganisms. *Pestalotiopsis* sp. (DO2) could inhibit the growth of *Bacillus subtilis* with the highest inhibition zone of about 20 mm.

Table 2. Endophytic fungi isolated from Dipterocapous plants.

Endophytic fungi	Classification	Plant species				Total
		DT	SO	SS	DO	
Phyllosticta spp.	Coelomycetes	-	-	8	7	**15**
Pestalotiopsis sp.	Coelomycetes	2	-	-	3	**5**
Fusarium sp.	Hyphomycetes	-	-	2	-	**2**
Nodulisporium spp.	Hyphomycetes	10	3	-	-	**13**
Paecilomyces sp.	Hyphomycetes	-	-	-	5	**5**
Penicillium sp.	Hyphomycetes	-	2	2	1	**5**
Phomopsis sp.	Coelomycetes	2	3	3	-	**8**
Xylaria sp.1	Ascomycetes	3	4	-	3	**10**
Xylaria sp.2	Ascomycetes	-	-	1	-	**1**
Xylaria sp.3	Ascomycetes	-	-	-	2	**2**
Daldinia sp.	Ascomycetes	-	2	-	-	**2**
Total		**17**	**14**	**16**	**21**	**68**

These results are in agreement with the results obtained for endophytic fungi isolated from teak (*Tectona grandis* L.) and rain tree (*Samanea saman* Merr.) leaves (Chareprasert *et al*., 2006), which found that the endophytic fungi from teak and rain tree could inhibit the growth of Gram positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* to a greater degree than Gram negative bacteria (*Escherichia coli*). Lumyong and Boonjim (1998) also reported that endophytic fungal strains E 4.11 and E 4.22 isolated from *Lithocarpus* sp. could produce some metabolite active against *Staphylococcus aureus*. *Xylaria* sp.1 showed the highest broad spectrum of antimicrobial activity against all test microorganisms. *Xylaria* spp., which are common endophytic inhabitants of most tropical plants investigated (Liu *et al*., 2008) have been previously investigated

for their production of new metabolites and have proven to be a good source of bioactive compounds (Espada *et al*., 1997). There are very few reports, however, on endophytic fungi isolated from Dipterocarpous plants in Thailand.

Further investigation of endophytic fungi is considered to be importance since they hold promise as a source of new drugs and novel bioactive metabolites with a high activity against pathogenic microorganisms.

Table 3. Inhibition zones resulting from crude extracts against the test microorganisms.

Endophytic fungi	Inhibition zone (mm)				
	BACIL	STAPH	PSEUD	ESCHE	CAND
Phyllosticta sp.SS6	-	-	-	-	-
Phyllosticta sp.DO7	-	-	-	-	-
Pestalotiopsis sp.DT1	14.6±0.5	10.8±0.2	-	-	-
Pestalotiopsis sp.DO2	20.1±0.7	14.6±0.5	-	6.8±0.2	-
Nodulisporium sp.DT6	15.8±0.2	12.6±0.5	8.3±0.5	9.3±0.5	16.3±0.5
Nodulisporium sp.SO4	8.8±0.2	9.1±0.2	8.1±0.2	-	-
Furarium sp.SS1	7.3±0.5	10.6.±0.5	-	-	-
Phomopsis sp.DT3	6.3±0.5	-	-	-	-
Phomopsis sp.SS4	-	-	-	-	-
Phomopsis sp.SO1	6.3±0.5	-	-	-	-
Paecilomyces sp.DO5	11.6±0.5	-	-	-	-
Penicillium sp.SO3	11.5±0.5	8.3±0.5	-	-	-
Penicillium sp.SS10	8.6±0.5	-	-	-	-
Penicillium sp.DO4	8.0±0.8	-	-	-	-
Xylaria sp.1 DT10	12.3±0.5	9.5±0.2	8.5±0.2	7.6±0.5	-
Xylaria sp.1 SO7	13.6±0.5	9.1±0.7	-	-	-
Xylaria sp.1 DO9	7.3±0.5	10.3±0.5	8.3±0.5	6.5±0.7	17.6±0.5
Xylaria sp.2 SS8	19.3±0.5	13.5±0.5	-	-	-
Xylaria sp.3 DO18	6.6±0.4	7.1±0.2	7.0±0	6.2±0.2	-
Daldinia sp.SO14	12.3 ±0.5	8.6±0.5	6.0±0	-	-

Values are mean (±SE) of three replications.

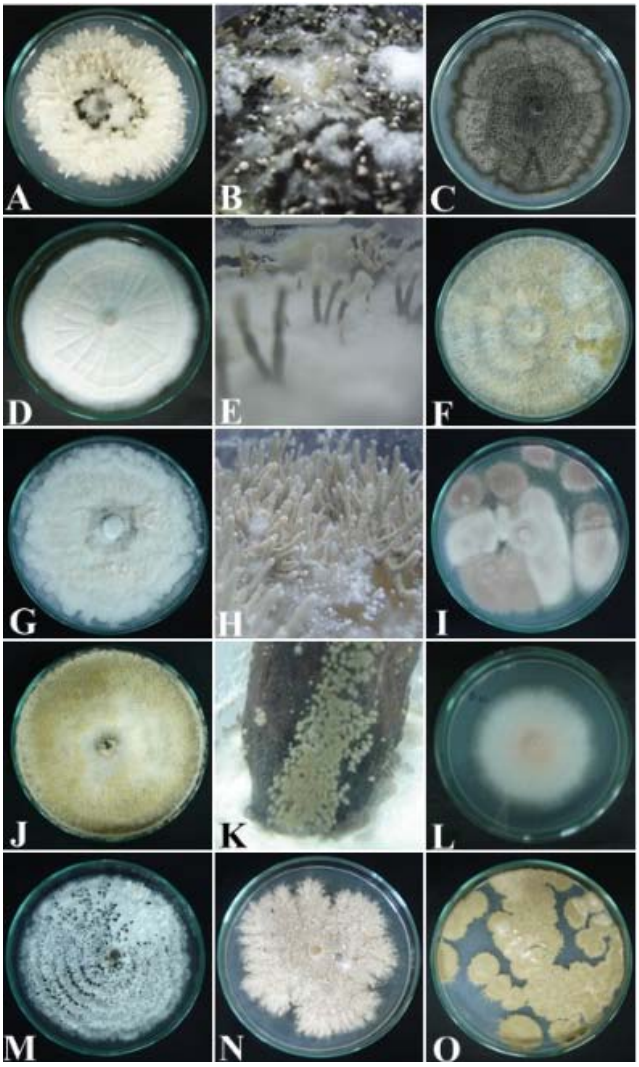

Fig. 1. Endophytic fungi of different morphotaxa; A and B (*Xylaria* sp.1), C (*Phyllosticta* spp.), D and E (*Xylaria* sp.2), F (*Nodulisporium* spp.), G and H (*Xylaria* sp.3), I (*Paecilomyces* sp.), J and K (*Daldinia* sp.), L (*Fusarium* sp.), M (*Pestalotiopsis* sp.), N (*Phomopsis* sp.) and O (*Penicillium* sp.).

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