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Communication

Isolation and primary identification of endophytic fungi from *Cephalotaxus mannii* trees

Pramuan Saithong [1,*], Wanchai Panthavee [1], Siriporn Stonsaovapak [1] and Li Congfa [2]

[1] Department of Applied Microbiology, Institute of Food Research and Product Development, Kasetsart University, 50 Phaholyothin Rd., Chatuchak, Bangkok 10900, Thailand

[2] Department of Food Science and Engineering, College of Science and Engineering, South China University of Tropical Agriculture (SCUTA), Hainan, China

* Corresponding author, e-mail: ifrpmst@ku.ac.th

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Abstract: Fifty-two isolates of endophytic fungi were collected from the bark of *Cephalotaxus mannii* (plum-yew) trees located in the north of Thailand and the south of China. All isolates were identified based on colony morphology and examination of spores and fruiting bodies using stereo and light microscopes. Thirty-five isolates (67.3%) belonging to 13 genera were recorded, viz. *Cladosporium* sp., *Acremonium* sp., *Trichoderma* sp., *Monilia* sp., *Fusarium* sp., *Spicaria* sp., *Humicola* sp., *Rhizoctonia* sp., *Cephalosporium* sp., *Botrytis* sp., *Penicillium* sp., *Chalaropsis* sp. and *Geotrichum* sp., while 17 strains (32.7%) were unidentified. The dominant genera found both in northern Thailand and southern China were *Acremonium* sp., *Monilia* sp. and *Fusarium* sp. *Cladosporium* sp. and *Trichoderma* sp. were found only in southern China, whereas *Spicaria* sp., *Humicula* sp., *Rhizoctonia* sp., *Botrytis* sp., *Penicillium* sp., *Geotrichum* sp., *Chalaropsis* sp. and *Cephalosporium* sp. were found only in northern Thailand. Thus, there seemed to be a significant difference in the genera of endophytic fungi from *Cephalotaxus mannii* trees of different sources.

Keywords: *Cephalotaxus mannii*, endophytic fungi, primary identification of fungi

INTRODUCTION

Endophytic fungi are found on all kinds of plants, i.e. trees, grasses, algae and herbaceous plants. Endophytic fungi live within a plant's tissue without causing any symptoms or apparent injury to the host. The colonisation of plant tissues by endophytic fungi occurs in a manner similar to those of plant pathogens and mycorrhizae [1]. Colonisation comprises a sequence of steps involving host recognition by the fungus, spore germination, penetration of the epidermis, and tissue colonisation.

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Most endophytic fungi belong to ascomycetes and fungi imperfecti [2]. The equilibrium between the host and the fungi seems to be controlled in part by chemical factors, for example herbicidal natural products produced by the fungi versus antifungal metabolites synthesised by the host plants [2]. There are also reports demonstrating that many antitumour agents such as taxol [3-4] as well as antimicrobial agents [5-7] can be produced by these endophytic fungi. The genus *Acremonium* sp. was reported by Jeamjitt et al. [8] to be especially capable of producing secondary metabolites. These substances may have applications outside the host plant in which they normally reside. Endophytic fungi have been primarily studied in temperate regions; tropical endophytic fungi are less well understood. Fungi from this unique habitat have been shown to be the sources of new species, possibly containing new bioactive compounds.

Endophytic fungi of tropical plants have been widely researched in Thailand [8-14]. These previous studies are exemplified in Table 1. However, endophytic fungi from *Cephalotaxus mannii* have not been well researched in Thailand. *Cephalotaxus mannii* is a kind of pharmaceutical plant and is well known as a rich source of endophytic fungi. It is mainly distributed in South-East Asia and China, especially in northern Thailand [15]. Five alkaloids, namely harringtonine, isoharringtonine, homoharringtonine, dexoharringtonine and cephalezomines have been isolated from *Cephalotaxus mannii*, *C. sinensis, C. hainanensis*, *C. oliveri* and *C. harringtonia* var. *nana* respectively, and have been developed as clinical medicines for hospital treatment of leukemia patients [2, 6, 16].

Studies of useful endophytic fungi from such pharmaceutical plants will be of great value to ecology and pharmacology. The present study focuses on screening and identification of endophytic fungi isolated from *Cephalotaxus mannii* collected from two regions: northern Thailand (Chiang Mai and Chiang Rai provinces) and southern China (Hainan Island).

Table 1. Some studies on endophytes in Thailand [1]

Host	Number of taxa	Reference
Amomum siamense	33	[17]
Bambusa spp.	96	[18]
Musa sp. (banana)	61	[19]
Palms	39	[20]
Dimocarpus longan (longan)	18	[21]

MATERIALS AND METHODS

Sources of Endophytic Fungi

Bark samples of *Cephalotaxus mannii* were gathered from tropical rainforest complexes which have high tree species diversity during the rainy season in three locations in northern Thailand (Doi Tung, Chiang Rai province, and Doi Inthanon and Doi Suthep-Pui, Chiang Mai province) and from Hainan Island, off the southern coast of China. All samples were collected at an altitude of approximately 800 metres above sea level. Table 2 shows the numbers of samples and the locations where they were collected. These bark samples were cross-sectioned at different parts of the trees (low,

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middle and high) using sterilised knives to obtain pieces of 2.0-3.0×1.5-2.0×0.3-0.5 cm (length×width×thickness). The samples were then sealed with Parafilm®, a flexible thermoplastic film, to prevent drying during transport and maintained at 4°C until further processing.

Isolation of Endophytic Fungi

Endophytic fungi isolation was carried out under aseptic condition. The outer bark of each sample was detached with a sterilised sharp blade, cleaned by washing with running tap water several times and soaked in 70% (v/v) ethanol for 10-20 min. It was then washed several times with sterilised water, dipped into 0.1% $HgCl_2$ for 1-2 min, again washed with sterilised water 3-5 times and then put into a beaker of sterilised distilled water. The bark sample was then cut into small pieces, each piece put on a plate of potato dextrose agar (PDA) medium supplemented with chloramphenicol (30 μg/ml) and streptomycin (30 μg/ml), and the plate cultivated at 30°C to promote fungal growth and sporulation. Individual hyphal tips of the fungus were then picked up from each plate, inoculated onto another PDA medium plate, and incubated at 30°C for at least 1 week. Each fungal culture was checked for purity and transferred to another agar plate using the hyphal tips. The purified fungal isolates were numbered, transferred separately to PDA slants, and kept at 4°C.

Table 2. Bark samples of *Cephalotaxus mannii* trees collected from various places

Location	Number of samples	Place
Near a canal on the mountain	1	Doi Suthep-Pui National Park, Chiang Mai, Thailand
Top of the mountain Near a roadside in the park	3	Doi Tung (Wat Phra That Doi Tung) Chiang Rai, Thailand
Near a roadside in the park On a hillside in the forest Deep in the forest	11	Doi Inthanon National Park, Chiang Mai, Thailand
Top of the mountain Top of the mountain	2	Diao Luo Mountain, Hainan Island, China
Foot of the mountain	2	Qi Xian Ling Mountain, Hainan Island, China
Top of the mountain	1	Jian Feng Ling Mountain, Hainan Island, China

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Identification of Endophytic Fungi

For characterisation of the morphology of fungal isolates, slides prepared from cultures were stained with bromothymol blue reagent and examined with a bright-field and phase-contrast microscope. Identification was based on morphological characteristics such as growth pattern, hyphae, colour of colony and medium, surface texture, margin character, aerial mycelium, mechanism of spore production and characteristics of the spore [22]. An Olympus BH-2 with Nomarski interference contrast was employed for examination by light microscopy.

RESULTS

Fifty-two isolates of endophytic fungi were collected from 20 samples (Table 2). All endophytic fungi could be cultivated on artificial media and maintained as a pure culture. They exhibited characteristic colony and microscopic morphology that could be used to differentiate them. Most of them belonged to ascomycetes and fungi imperfecti, as shown in Table 3. Some results of characterisation of colony and microscopic morphological study are shown in Figures 1 and 2 respectively. All isolates were identified as belonging to 13 genera, namely *Cladosporium* sp., *Acremonium* sp., *Trichoderma* sp., *Monilia* sp., *Fusarium* sp., *Spicaria* sp., *Humicula* sp., *Rhizoctonia* sp., *Cephalosporium* sp., *Botrytis* sp., *Penicillium* sp., *Chalaropsis* sp. and *Geotrichum* sp. (Table 3). The dominant genus found in the north of Thailand was *Geotrichum* sp. (7 isolates, 20.0%) while *Trichoderma* sp. was the dominant genus found in the south of China (4 isolates, 23.5%). *Cladosporium* sp. and *Trichoderma* sp. were found only in the south of China while *Spicaria* sp., *Humicula* sp., *Rhizoctonia* sp., *Botrytis* sp., *Penicillium* sp., *Geotrichum* sp., *Chalaropsis* sp. and *Cephalosporium* sp. were found only in the north of Thailand. Genera common to northern Thailand and southern China consisted of *Acremonium* sp., *Monilia* sp. and *Fusarium* sp. Some fungal isolates belonged to rare genera, i.e. *Spicaria* sp., *Humicula* sp. and *Acremonium* sp. Some fungi, viz. 5 isolates (29.4%) from southern China and 12 isolates (34.3%) from northern Thailand, could not be identified due to lack of spore formation. These results indicated that there are significant difference in the genera of endophytic fungi from *Cephalotaxus mannii* trees of different locations.

Huang et al. [2] reported that endophytic fungi can produce antitumour or antifungal activities. These fungi were isolated from the inner bark of three kinds of pharmaceutical plants including *Cephalotaxus* sp., and were identified as belonging to six genera, namely *Paecilomyces* sp., *Cephalosporium* sp., *Mortierella* sp., *Mucor* sp., *Trichoderma* sp. and *Cladosporium* sp.

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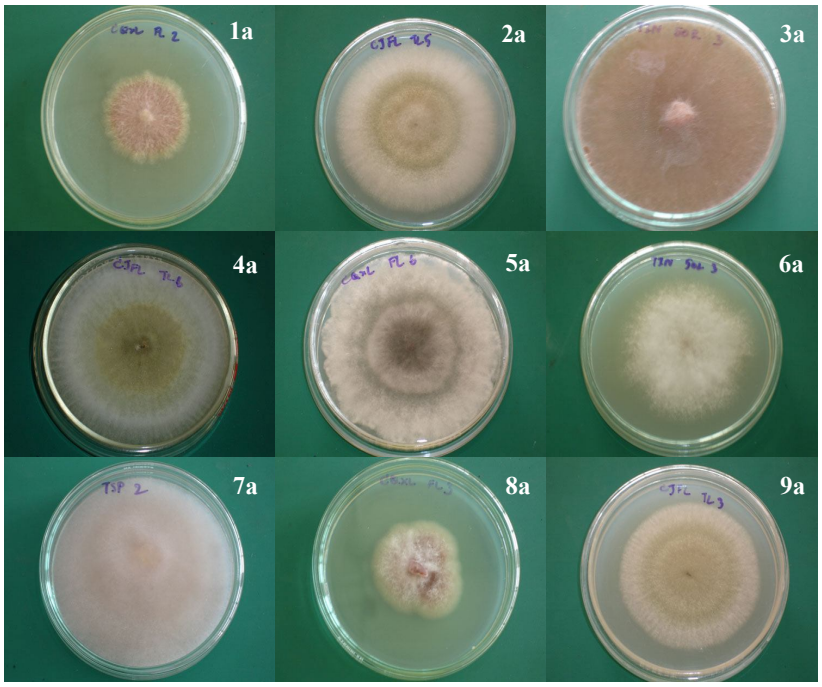

Figure 1. Colour variation among colonies on PDA medium plates at 3 days: (1a) unidentified strains; (2a) *Botrytis* sp.; (3a) *Geotrichum* sp.; (4a) *Botrytis* sp.; (5a) *Acremonium* sp.; (6a) unidentified strains; (7a) *Geotrichum* sp.; (8a) *Fusarium* sp.; (9a) *Botrytis* sp.

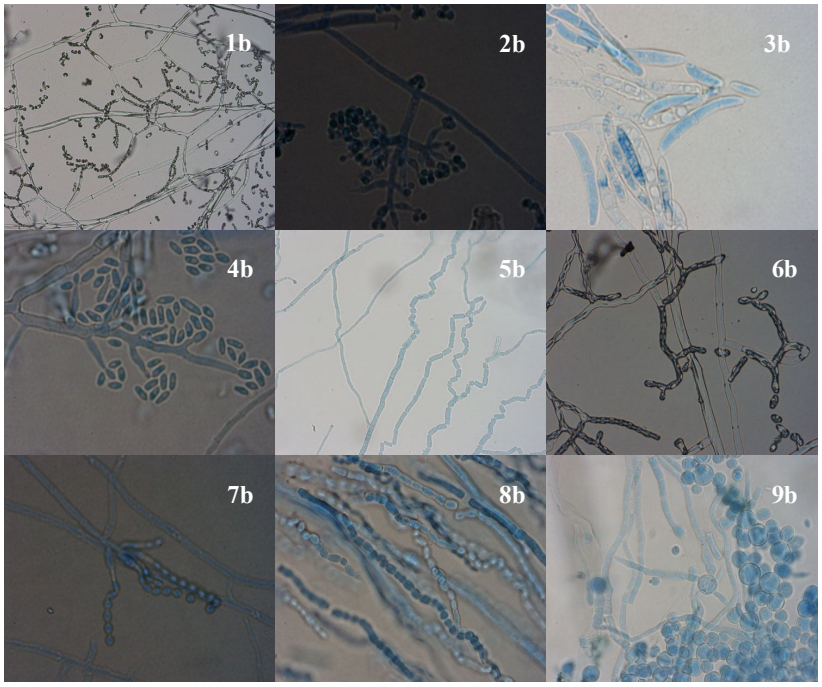

Figure 2. Light microscopic observation of fungi: (1b) *Monilia* sp.; (2b) *Trichoderma* sp.; (3b) *Fusarium* sp.; (4b) *Cephalosporium* sp.; (5b) *Geotrichum* sp.; (6b) *Monili*a sp.; (7b) *Spicaria* sp.; (8b) *Geotrichum* sp.; (9b) unidentified strain

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Table 3. Genera of isolated endophytic fungi

Genus	Number of endophytes from *Cephalotaxus* sp. trees at different locations			
	Doi Tung	Doi Inthanon	Doi Suthep-Pui	Hainan Island
Cladosporium sp.				1
Acremonium sp.	2			3
Trichoderma sp.				4
Monilia sp.	1	1		1
Fusarium sp.		1		3
Spicaria sp.	1			
Humicula sp.	2	1		
Rhizoctonia sp.		1		
Cephalosporium sp.		1		
Geotrichum sp.		5	2	
Botrytis sp.		2		
Penicillium sp.		2		
Chalaropsis sp.		1		
unidentified strains	4	8		5

CONCLUSIONS

Endophytic fungi from the *Cephalotaxus mannii* trees were isolated and identified by their morphology and spore characteristics. From twenty samples from northern Thailand and southern China were obtained 52 isolates, which were identified as belonging to 13 genera with some unidentified strains. *Geotrichum* sp. and *Trichoderma* sp. were found to be the dominant genera in northern Thailand and southern China respectively. Bark samples from Doi Inthanon (northern Thailand) had the highest number of fungi (15 isolates and 8 unidentified, 44.2%) while those from Doi Suthep-Pui (northern Thailand) had the fewest (2 isolates, 3.8%). *Cladosporium* sp. and *Trichoderma* sp. were found only in the south of China while *Spicaria* sp., *Humicula* sp., *Rhizoctonia* sp., *Botrytis* sp., *Penicillium* sp., *Geotrichum* sp., *Chalaropsis* sp. and *Cephalosporium* sp. were found only in the north of Thailand. Some isolates belonged to the rare genera, namely *Spicaria* sp., *Humicula* sp. and *Acremonium* sp. However, these results should be considered as those of an initial study. Further investigation, e.g. 18S rDNA sequence comparisons (molecular techniques), is required to confirm the classification of these isolates.

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