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Contributed Paper

Diversity of Endophytic Fungi Associated with *Cinnamomum bejolghota* (Lauraceae) in Northern Thailand

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ABSTRACT

A total of 2,774 culturable endophytic fungi were isolated from 2,250 samples of healthy leaves (vein and intervein) and stems of mature plants of wild cinnamon tree, *Cinnamomum bejolghota* collected from Doi Suthep Pui National Park, northern Thailand. Colonization rates were high ranging from 94.8% to 99.7% at the three sample sites in the wet and dry seasons. Endophytes were most frequently isolated from tree in the wet season at the Doi Suthep Pui Peak site. Species richness was consistently higher in the wet season. *Colletotrichum gloeosporioides* and *Phomopsis* spp. were the most common endophytic fungi in the leaves and stems at all sites. All isolated endophytes were classified into 9 ascomycetes and 19 anamorphic taxa (6 coelomycetes and 13 hyphomycetes) using conventional methods. *Colletotrichum gloeosporioides*, *C. acutatum*, *Phomopsis* spp., *Guignardia mangiferae* and xylariaceous taxa were found consistently dominant.

Keywords: endophytic fungi, cinnamon tree, Thai medicinal plant

1. INTRODUCTION

Endophytes are microbes that colonize living internal tissues of plants without causing any harm to their host [1-2]. Many recent studies have revealed the ubiquity of these fungi, with an estimate of at least one million species of endophytic fungi residing in plants [3]. To date, only about 80,000-100,000 fungal species have been described [4-5], out of a conservative estimate of 1.5 million [6]. Endophytes protect their hosts from infectious

agents and adverse conditions by secreting bioactive secondary metabolites [7-9]. The endophytic fungi play important physiological [10] and ecological [11-12] roles in their host life. A variety of relationships exist between fungal endophytes and host plants ranging from mutualistic or symbiotic to antagonistic or slightly pathogenic [13-15]. Additionally, endophytic fungi represent an important and quantifiable component of fungal biodiversity

and are known to affect plant community diversity and structure [16-17]. There is ongoing interest in the biodiversity of endophytic fungi in Thailand [18-22]. There is enormous potential for exploiting these fungi for medicinal, agricultural and industrial uses [23-24]. Recently, medicinal plants have been found to harbor endophytic fungi that are believed to be associated with the production of pharmaceutical products [25-26]. Much research had been carried out to characterize endophytic fungi residing in medicinal plants [27-29].

The present study was initiated in order to investigate the diversity of endophytic fungi in the different tissues, seasons and sites of wild cinnamon tree, *Cinnamomum bejolghota* (Buch.-Ham.) Sweet. from northern Thailand.

2. MATERIAL AND METHODS

2.1 Sample Collection

Samples were randomly collected from ten plants of *C. bejolghota* at three localities in Doi Suthep Pui National Park, northern Thailand. Collections were made at Monthatarn waterfall (18°49'3"N, 98°55'27"E and altitudes 700 m), Medicinal Plant Garden (18°48'22"N, 98°54'51"E and altitudes 1076 m) and Doi Suthep Pui Peak (18°48'26"N, 98°53'48"E and altitudes 1500 m), in February 2008 (dry season, temperature 28-33°C, humidity 56-79%, wind speed 3 km h⁻¹ and scattered cloudy) and May 2008 (wet season, temperature 26-31°C, humidity 80-100%, wind speed 3 km h⁻¹ and mostly cloudy). Five branches (2 cm diam) and 10 leaves from each plant were collected. The samples were brought to the laboratory and processed within 24 h.

2.2 Isolation of Endophytic Fungi

Samples were washed in running tap water for 15 min. 150 leaf discs (5 mm diam)

comprising 75 discs including vein tissues and 75 discs incorporating leaf tissue only plus 75 stem sections (10 mm long) were randomly cut from the leaves and stems. In total 750 leaf samples and 375 stem samples were isolated. All leaf tissue discs were surface sterilized in 75% ethanol for 30 s, 2% sodium hypochlorite for 3 min and 95% ethanol for 30 s under a laminar flow hood [30]. The sterilized samples were placed in Petri dishes containing 2% malt extract agar, 0.05% streptomycin sulfate and 0.03% rose bengal. Petri dishes were sealed with parafilm and incubated at room temperature (25±2°C) for one week. The fungi growing out from the samples were aseptically transferred to two culture media, potato dextrose agar (PDA) and malt extract agar (MA). The fungal isolates were maintained on corn meal agar (CMA) slants. Various methods were applied to stimulate spore production [31].

2.3 Identification of Endophytic Fungi

The endophytic fungi were identified based on their morphotaxonomic characters [32-38]. All identified endophytic fungi were maintained in cryovials at -20°C in a deep freezer. All fungal isolates were deposited at Research Laboratory for Excellence in Sustainable Development of Biological Resources, Faculty of Science, Chiang Mai University or at the BIOTEC Culture Collection, Bangkok, Thailand.

2.4 Data Analysis

The overall colonization rates (%CR) were expressed in percentage, $CR = (N_{COL} / Nt) \times 100$, where N_{COL} = total number of segments in a sample yielding ≥ 1 isolate; Nt = total number of segments in that trial [39]. Intensity was also calculated and used to demonstrate the degree of multiple colonization from the samples in different trials, $IT = (N_{NUM} / Nt) \times 100$, N_{NUM} = total

number of isolates yielded in a given trial; Nt = total number of segments in that trial [20]. The Simpson's diversity index (D) and Shannon-Wiener index (H') were calculated (Shannon & Weaver 1949; Simpson 1949). Correspondence analysis was used to analyze the tissue unit and fungal species ordinations to verify the ecological interrelationships between tissue units and fungal species in a single analysis. JMP software was used to carry out the correspondence analysis [40].

3. RESULTS AND DISCUSSION

3.1 Overall Prevalence and Intensity

A total of 2,774 endophytic isolates were recovered from 2,250 plant tissues of *Cinnamomum bejolghota* collected from three different sites in wet and dry seasons. They were identified into 28 taxa comprising 9 ascomycetes and 19 anamorphic taxa (6 coelomycetes and 13 hyphomycetes). The overall colonization rates (%) and intensity of assemblages of endophytes recovered at each site in wet and dry seasons are given in (Table 1). There was no significant difference between isolate prevalence at each season or site. Twenty-eight taxa of endophytic fungi

were isolated with colonization rates at 94.8-99.7%. The results were within the range of many host plants studies in tropical regions. Colonization rate of endophytic fungi in traditional Chinese medicinal plants ranged from 2-100% [28, 41-42]. The variation in colonization rates from tropical palm endophytes was reported as 0.0-98.0% [22, 43-45]. In addition, the colonization rate in studies on banana endophytes was reported to range from 29.6-67% [21-25]. The colonization rate from *Amomum siamense* reported by Bussaban *et al.* [20] varied from 70-83% and Rakotoniriana *et al.* [46] showed that the overall foliar colonization rate of *Centella asiatica* collected in Madagascar was 78%.

The taxa at each site in different seasons were presented in Table 2. *Colletotrichum gloeosporioides*, *Phomopsis* spp. and *Guignardia mangiferae*, which were isolated frequently in this study, have been reported as endophytes in a wide range of hosts in the tropics [8, 20-22, 27-28, 41, 46-50]. In addition, Bussaban *et al.* [20] and Gond *et al.* [47] indicated that most fungal endophytes belong to the hyphomycetes. In this study, hyphomycetes were more abundant than coelomycetes and

Table 1. Summary data of fungal endophytes diversity from *Cinnamomum bejolghota*.

Characteristic	Wet season			Dry season		
	MON	MED	SUT	MON	MED	SUT
No. of sample	375	375	375	375	375	375
No. of isolates recovered	480	511	522	449	390	422
Intensity (No. of isolates per sample)	1.276	1.244	1.261	1.192	1.063	1.132
Colonization rates (%)	99.7	94.8	96.8	99.3	98.2	97.8
Species richness (S)	18	21	21	14	12	15
Simpson's index (D)	0.205	0.215	0.186	0.238	0.333	0.390
Simpson's index of diversity (1-D)	0.795	0.795	0.814	0.761	0.667	0.710
Shannon index of diversity (H')	2.088	2.143	2.305	1.924	1.598	1.790

MON= Monthatharn waterfall, MED= Medicinal Plant Garden and SUT= Doi Suthep Pui Peak.

others. Interestingly, one of the mycelia sterilia was identified to *Muscodora cinnamomi* which produces volatile organic compounds [51].

3.2 Effect of Season and Site

The three sites were sampled over two seasons to determine the fungal endophytic colonization rate. Samples taken from the

Table 2. Colonization rates of fungal endophytes isolated from *Cinnamomum bejolghota* at each site in the wet and dry season.

Taxa	Wet season			Dry season			Plant part		
	MON	MED	SUT	MON	MED	SUT	Vein	Inter vein	Stem
<i>Curvularia lunata</i>				2.8					1.0
<i>Chaetomium globosum</i>	3.1	0.5	2.1	2.6		1.4	0.2	1.1	1.9
<i>Cladosporium tenuissimum</i>	6.2		2.2	2.8		1.6	1.8	1.2	1.2
<i>Colletotrichum acutatum</i>	3.5	8.6	8.6	7.5	7.9	7.9	5.9	4.7	1.9
<i>Colletotrichum coccodes</i>		1.1	2.4				1.2		
<i>Colletotrichum gloeosporioides</i>	35.2	11.1	10.5	42.5	54.6	13.9	18.9	18.5	13.3
<i>Eupenicillium shearii</i>	1.8	0.9	3.6	2.8				2.1	1.1
<i>Fusarium solani</i>		1.5	0.7	1.7		1.4	1.7		
<i>Fusarium</i> sp. 1		1.3					0.4		
<i>Gaeumannomyces graminis</i>						1.1		0.3	
<i>Glomerella</i> spp.	2.2	3.5	1.7	2.1	0.7	1.4	2.1	1.1	
<i>Guignardia mangiferae</i>	2.2	42.2	6.5	4.2	10.7	50.2	11.2	12.1	13.2
<i>Humicola</i> sp.	1.2		0.9	1.1				1.1	
<i>Leiosphaerella</i> sp.		0.5							0.1
<i>Muscodora cinnamomi</i>			0.7					0.2	
<i>Nectria</i> sp.	2.8							0.8	
<i>Nigrospora oryzae</i>			1.1				0.4		
<i>Nodulisporium</i> spp.	1.2	1.3	2.1	1.5	1.2	1.6	2.6		0.5
<i>Oidiodendron</i> sp.	1.1						0.4		
<i>Periconia</i> sp.		0.7				1.4	0.2	0.4	
<i>Pestalotiopsis</i> spp.	1.1	1.1	2.1				0.6		0.8
<i>Phomopsis</i> spp.	26.2	10.7	39.2	20.7	11.5	8.5	9.4	10.1	16.7
<i>Phoma</i> spp.	1.2	1.5	1.7		0.7		1.1	0.4	0.2
<i>Sclerotium</i> sp.		0.5			1.2			0.3	0.2
<i>Talaromyces flavus</i>	1.2	1.3				0.9	0.4	0.4	0.2
<i>Torula</i> spp.			1.7					0.6	
<i>Trichoderma</i> spp.	1.2	1.7	1.1		2.8	0.9		1.5	0.9
<i>Verticillium</i> sp.		1.5	1.3		2.1				1.6
Xylariaceous taxa	5.4	3.5	4.5	4.4	4.1	4.2	2.8	2.5	3.1
Mycelia sterilia	2.9	2.9	4.4	2.6	2.1	3.2	2.2	2.3	1.5
Total	99.7	97.9	99.1	99.3	99.6	99.5	63.5	61.7	59.4

MON=Monthatharn waterfall, MED=Medicinal Plant Garden and SUT= Doi Suthep Pui Peak.

tree in Monthatharn waterfall showed the highest percentage colonization rates in wet season and lowest percentage colonization rates in dry season (Table 2). The colonization rate and intensity of endophytic fungi in wet and dry seasons were similar. According to the study of endophytic fungi in *Amomum siamense* [20] and the palms *Calamus kerrianus* and *Wallichia caryotoides* [22] from Doi Suthep Pui National Park, isolation prevalence in both wet and dry seasons was similar, perhaps because the study sites had high humidity, temperature and rainfall throughout the year.

The number of isolates recovered at the three sites in the wet season was also higher than in the dry season (Table 2). The total number of endophyte species recovered from the tree in wet season located in Doi Suthep Pui Peak site was the highest, followed by Monthatharn waterfall and the Medicinal Plant Garden (Table 1). In the dry season, *Cinnamomum bejolghota* samples from Monthatharn waterfall and the Medicinal Plant Garden were most frequently colonized by *C. gloeosporioides*. Seasonality affects the number of isolates and species richness. The number of isolates recovered

and species richness were higher in the wet season, presumably because the wet conditions are favorable for fungal sporulation and infection. This causes endophyte infection to increase during the wet season [48-59]. A climatic factor may determine spread and germination success of endophytic fungal spores [53]. Furthermore, location within site also affected the endophytic species abundance. Several studies have shown that sites specific factor such as, elevation, humidity, density of canopy cover and innate host susceptibility may influence the level of fungal infection among sites [7, 20-22, 49, 54].

3.3 Effect of Tissue Type

There was no significant difference in the number of isolates recovered from vein (34.4%), internal vein (33.4%) and stem tissues (32.2%) (Figure 1). There was also no significant difference in the colonization rates between the vein and inter vein tissues. The colonization rate of taxa isolated from different tissues indicates that some endophytic taxa have an affinity with specific tissue types. *Colletotrichum gloeosporioides* was the most frequently isolated from leaf tissues and *Guignardia mangiferae* and *Phomopsis* spp. were the most commonly found in the stem

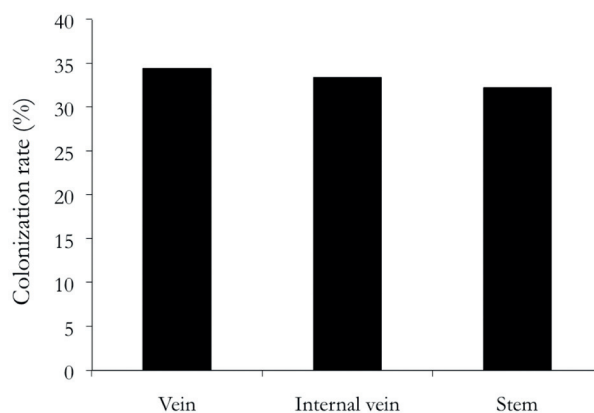


Figure 1. Colonization rates of endophytic fungi from *Cinnamomum bejolghota* tissues.

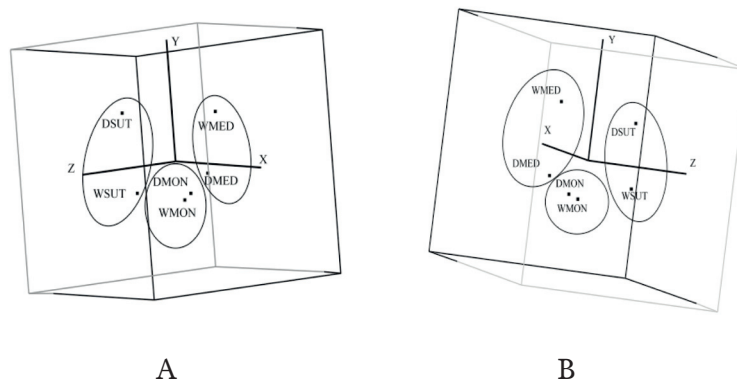
tissues (Table 2). Several studies have also shown that stem, twig or petiole tissues had a relatively high frequency of many fungal species [21-22, 27, 41, 47, 55]. However, the present study found that leaf tissues had a higher colonization rate than stems, suggesting that multiple infections of the leaves by endophytes are a common phenomenon [20, 28, 48, 50]. The dominance of endophytic fungal species found in stems and leaves were different. *Guignardia mangiferae* and *Phomopsis* spp. were found to be dominant in stems whereas *C. gloeosporioides* was dominant in leaves. In addition, *Curvularia lunata*, *Leiosphaerella* sp. and *Verticillium* sp. were isolated only from stem tissues and *C. coccoodes* was only isolated from vein tissue (Table 2).

3.4 Statistical Analysis

The number of isolates ranged from 480 to 522 in the wet season and 390 to 449 in the dry season. The species richness (S) in the wet season was higher than in dry season. Doi Suthep Pui Peak and Medicinal Plant Garden had the overall highest number of 21 taxa in each site in wet season. In dry season, Monthatharn waterfall showed the highest of an overall number of endophytes

(Table 1). The Shannon and Simpson diversity indices were highest in the Doi Suthep Pui Peak site ($H' = 2.305$; $1-D = 0.814$) and lowest in Monthatharn waterfall ($H' = 2.088$; $1-D = 0.795$) for the wet season. In addition, the data in dry season showed the highest diversity at Monthatharn waterfall ($H' = 1.924$; $1-D = 0.761$) and lowest in the Medicinal Plant Garden ($H' = 1.598$; $1-D = 0.667$) (Table 1).

Three-dimensional correspondence analysis was performed to investigate patterns of endophyte assemblages across seasons and sites on *C. bejolghota* (Figures 2). The three principal axes accounted for 100% of the variability in the data matrix. The x-axis separates the two seasons (wet and dry), while the z-axis separated the three sites, indicating that there are preferential seasons and sites for fungi on this plant. The result of the analysis also showed that the seasons and sites affected fungal endophyte communities. Only fungal communities of *C. bejolghota* collected from Monthatharn waterfall site in the wet season (WMON) were not differentiated from the dry season (DMON), indicated by the closer distances between the two samples.



Figures 2. Three-dimensional correspondence ordination of endophytic communities of *Cinnamomum bejolghota*. **A.** Diagram oriented at x- and y-axes. **B.** Diagram oriented at z- and y-axes. Season and site are indicated by: W= wet season, D= dry season, MON= Monthatharn waterfall, MED= Medicinal Plant Garden and SUT= Doi Suthep Pui Peak.

4. CONCLUSION

The endophytes isolated in this study were obtained by traditional methodology, which involved transferring hyphal tips growing out of tissues and plating them out. The data obtained in the present study indicate the diversity of fungal endophytes exist in wild cinnamon tree, *Cinnamomum bejolghota* in northern Thailand. Total 2,774 isolates recovered were assigned to 28 taxa comprising 9 ascomycetes, 6 coelomycetes and 13 hyphomycetes. *Colletotrichum gloeosporioides*, *Phomopsis* spp. and *Guignardia mangiferae* and xylariaceous forms were found to be dominant regardless of the site, season and tissues. Although colonization rate and the intensity of endophytic fungi were similar in wet and dry seasons, generally diversity of endophytes was affected by season and site.

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