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Invited Paper

Endophytic Fungi from *Calamus kerrianus* and *Wallichia caryotoides* (Arecaceae) at Doi Suthep-Pui National Park, Thailand

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ABSTRACT

Fungal endophytes associated with the palms, *Calamus kerrianus* (rattan) and *Wallichia caryotoides* (taorang) were investigated at two sites within Doi Suthep-Pui National Park, Thailand. Endophytic fungi were isolated from different tissue types (petiole, leaf, lamina and leaf veins) during three periods of the year, rainy season (July-October, 1999), cold season (November 1999-February 2000) and hot season (March-June 2000). Thirty-five endophytic fungi isolated included xylariaceous taxa (20 morphotypes), sterile mycelia, one unidentified and 13 mitosporic fungi including *Cladosporium* sp., *Colletotrichum gloeosporioides*, *Corynespora*-like sp., *Fusarium* sp., *Guignardia cocaicola*, *Paecilomyces* sp. *Pestalotiopsis* sp., *Phialophora* sp., *Phoma* sp., *Phoma*-like sp., *Phomopsis* sp., *Phyllosticta* sp., and *Sarcopodium* sp. The endophyte species, their relative frequency, isolate prevalence and diversity did not differ significantly between host species, tissue types, study sites and seasons.

1. INTRODUCTION

The term 'endophyte' was introduced by de Bary in 1866, and applied to organisms that live within plant tissues [1]. The term includs all organisms that live symptomlessly within plant tissues at some period of their life cycle [2,3]. Endophytes can be pathogens or symbionts, and may also be described as mutualistic, [4,5]. Endophytic fungi are important for fungal diversity since they had an affect on structure and community of plants [6-9].

However, colonization or infection by endophytic organisms cannot be considered as causing disease, because a plant disease is an interaction between the host, parasite, vector and environment and symptoms are a result from the interaction [10]. Many bacteria as well as fungi are known to live endophytically [2]. Fungal endophytes have been investigated most intensively in the last decades, following the demonstration of symptomless ascomycetes and mitosporic fungi in European conifer needles [11]. Fungal endophytes may provide protection to their hosts. For example, fungi in redwood may function as antagonists to pathogens [12], while *Acremonium* endophytes of grasses, such as tall fescue and perennial ryegrass, may confer

increased drought tolerance to their hosts. The grass endophytic fungi interaction also produces various chemicals such as ergot alkaloids, tremogenic neurotoxins and paramine [13].

There have been more studies on the endophytic fungi in temperate regions than in the tropical regions [14] However, interest in these fungi in the tropics is increasing [14], partly because of increasing fungal knowledge by local mycologists and the higher number of plant species compared to temperate regions [1,15]. Rodrigues [16,17] studied fungal endophytes in the foliage of *Euterpe oleraceae* (Amazonian palm) growing in the Brazilian Amazon estuary, and in Amazonian floodplains. Fr□hlich *et al.*=[18] examined endophytic fungi in *Licuala* sp. From from Brunei Darussalam and *L. ramsayi* from Queensland, Australia, while Taylor *et al.* [19] looked at those associated with the palm *Trachycarpus fortunei*, both within and outside its natural geographic range in China and New Zealand.

SContinuously studies (2000-present) on endophytic fungi from tropical and temperate native forests (2000-present) have provided data that support the higher estimation of fungal taxa than reported before the year 2000 [20,21]. In the present study endophytic fungi were isolated from *Calamus kerrianus* Becc. and *Wallichia caryotoides* Roxb. to determine the biodiversity of fungi within these two palms, and to determine if there is any difference in fungal communities between two sites at Doi Suthep-Pui National Park over three seasons: rainy season, cold season and hot season.

2. MATERIALS AND METHODS

2.1 Collecting Sites

Calamus kerrianus and *Wallichia caryotoides* were collected from forests at Huay Kog Maa (1000 m above sea level) and Medicinal Plant Garden (930 m above sea level) .in Doi Suthep-Pui National Park, Chiang Mai. Both palms are widely distributed in the two collection sites. Huay Kog Ma is an evergreen hill forest with much canopy, and the palms grow in association with other plant species. Medicinal Plant Garden is also an evergreen forest.

2.2 Sampling Procedure

Petioles and leaves of the palms (Figure 1) were sampled from healthy plants during three seasons, the rainy season (June-October, 1999), the cold season (November 1999-February 2000) and the hot season (March - May 2000). Ten plants from each species of palm were sampled from each site; Leaflets and petioles were cut and returned to laboratory in separate plastic bags. Samples were immediately washed in running water. A total of 50 petiole pieces (10 mm), 50 intervein leaf discs (5 mm), and 50 veinal leaf discs (5 mm) were randomly cut from each plant.

2.3 Surface Sterilization of Petioles and Leaves

Surface sterilization techniques followed Taylor *et al.* [19]. The concentration of sodium hypochlorite (NaOCl) and time were adjusted following a pilot experiment. Each leaf disc was surface sterilized by dipping in 95% ethanol for 60 seconds, then in a solution of 3% NaOCl for 8 minutes and finally in 95% ethanol for 30 seconds. Petioles were treated similarly, except they were dipped in 3% NaOCl for 15 minutes. After surface sterilization, tissues were dried on sterilized tissue paper and placed on half strength malt extract agar (MEA) containing 0.003% rose Bengal and 0.003% streptomycin sulfate. The rose Bengal was used to decrease the growth of faster developing fungi [18].

Petri dishes containing leaf discs and petiole fragments were incubated at 30°C for

4-8 weeks. The hyphae growing out from plant tissues were subcultured and transfer to plates containing MEA and incubated under near UV (nUV) light to promote fungal sporulation. Fungi were subcultured to MEA in microtubes and are kept at Department of Biology, faculty of Science, Chiang Mai University.

Sporulating fungi were identified to genus or species, using taxonomic keys [22-26]. Non sporulating isolates after 8 weeks were sorted into morphospecies on the basis of colony surface texture, hyphal pigmentation, exudates and growth rates as described by Fröhlich *et al.* [18].

2.4 Statistical Analysis

The isolation prevalence and intensity were calculated by using these formulae [27]

$$\text{Isolation prevalence} = \frac{\text{Total number of samples yielding at least 1 isolate}}{\text{Total number of samples in that trial}} \times 100$$

$$\text{Intensity} = \frac{\text{Total number of samples yielding isolates in a given trial}}{\text{Total number of samples in that trial}}$$

Isolation prevalence is expressed as a percentage [28] as commonly used in the literatures. Isolation intensity cannot be expressed as a percentage, but it is used to demonstrate the degree of multiple colonization from samples in different trials. A chi-square goodness-of-fit test was performed to test whether the isolation prevalence of twelve trials and isolation intensity of 36 trials were statistically different. Non parametric statistical tests were used because the data in most cases did not fit the assumptions for parametric statistics. A Kruskal-Wallis procedure was used for multisample analysis, such as the investigation of the number of isolates recovered from petiole, vein and intervein tissues for all plant samples at each site, and for the number of isolates recovered from different tissue types at each site in wet, dry and hot seasons [28]. In all analyses, P values are described as $P = 0.05$

3. RESULTS AND DISCUSSION

3.1 Isolation Prevalence

A total of 2619 fungal isolates were recovered from 1800 samples from the two palms species. Recovery rates varied between 20-94% (wet season), 10-98% (dry season) and 0-52% (hot season) (Table 1). The overall isolation prevalence (%) was not significantly different in each trial. These results are comparable with other studies of palm endophytes where the isolation prevalence has been reported. The colonization rate of *Licuala ramsayi* and *Licuala* sp. from Australia and Brunei was high at 80.8-89.2 % [22] whereas a lower rate (21-30%) was found from *Euterpe oleracea* [16]. Rodrigues and Samuels [29] reported a rate of only 12.5%. The colonization rate from *Trachycarpus fortunei* reported by Taylor *et al.* [1] varied from 23.4-57.3%.

Isolation prevalence in both wet and dry seasons, were comparatively high at both sites as compared to isolation prevalence in *Amomum siamense* [27]. They isolated endophytic fungi in the wet (August 1999) and dry (February 2000) seasons from the same two sites at Doi Suthep-Pui National Park [27]. Humidity and rain fall were high during the present study and may have influenced the colonization rate of fungal endophytes.

In the hot season the isolation prevalence in *Calamus kerrianus* and *Wallichia caryotoides* were low (Table 1). At this time of year most

of the palm leaves dried and were infected by pathogens. The newly expanded leaves were clean and looked healthy. Most samples collected during the hot season were of young petioles and leaves. The low isolation prevalence in the hot season may be due to the high temperature conditions. The successful infection from environment may be less since lower moisture condition. Taylor *et al.* [19] found that the isolation prevalence of endophytic fungi in *T. fortunei* declined with decreasing relative humidity and rainfall. Carroll and Carroll [30] demonstrated that Douglas fir was more heavily infected in moist sites than in dry sites and suggested that differences in elevation, humidity, density of canopy cover and innate host susceptibility were likely to cause the observed differences in endophytic infection between sites.

Leaf age may also affect results. Rodrigues

Table 1. The isolation prevalence (%) of endophytes from 50 samples of petiole segments, and dishes containing vein and interveins from *Calamus kerrianus* and *Wallichia caryotoides.*

	Wet season		Dry season		Hot season	
	HKM	**MPG**	**HKM**	**MPG**	**HKM**	**MPG**
Calamus kerrianus						
Petiole	82	74	84	10	44	34
Vein	54	94	82	72	24	0
Intervein	66	58	84	70	14	4
Wallichia caryotoides						
Petiole	76	64	80	92	52	48
Vein	88	64	94	98	22	42
Intervein	76	20	88	80	4	18

HKM = Huay Kog Maa Site, MPG = Medicinal Plant Garden Site.

[16] examined unopened leaves, newly expanded and mature leaves of *E. oleracea* and found significant variation endophytic fungi between all three classes in mature trees. Rodrigues and Samuels [29] suggested that the few endophytic taxa present in the tightly rolled spear leaf of *Licuala ramsayi* are systemic, and transmitted via the seed. By contrast, most endophytic fungi found in the expanded frond probably originated from airborne propagules. Fröhlich *et al.* [18] suggested that most endophytic fungi enter a plant by spores landing on a leaf surface and growing into the plant through the stoma or penetrating the host directly. The increased incidence of endophytic taxa in the older leaves may be due to the increased time of exposure, and hence increased accumulation of endophytic fungi from the environment. Older leaves also would have more time to accumulate transmitted colonizers that enter from the petioles. Passives entry and distribution of fungal spores into wood may be determined by anatomically structure of host and the environment change [31,32].

Factors that may contribute to changes in the endophytic community with leaf age, include weathering of the leaf cuticle, the presence of wounds, increased exposure to propagules with time, and changes in leaf physiology and chemistry [33-35].

3.2 Intensity

Intensity of endophytic fungi from two species of palms at different sites, tissue types and seasons were highest during wet season and lowest in the hot season (Table 2). The results of the Kruskal-Wallis test indicated that there were no significant differences between the numbers of fungal isolates recovered from petioles, intervein and vein tissues of either species of palm (Table 2). Thirty-five endophytic fungi were isolated and identified which included xylariaceous taxa (20 morphotypes), sterile mycelia, one unidentified and 13 mitosporic fungi including *Cladosporium* sp., *Colletotrichum gloeosporioides*, *Corynespora*-like sp., *Fusarium* sp., *Guignardia cocaicola*, *Paecilomyces* sp. *Pestalotiopsis* sp., *Phialophora* sp., *Phoma* sp., *Phoma*-like sp., *Phomopsis* sp., *Phyllosticta* sp., and *Sarcopodium* sp. Petiole tissue had a high relative frequency of many fungi, although the frequency of some fungi in vein or intervein tissues, were higher than in petiole (Table 3). For *C. kerrianus* the frequency of sterile mycelia, *Phomopsis* sp., *Xylaria* sp. 2, 3, 4 and 9 were higher from petiole tissues than from vein and intervein tissues. The frequency of *Collectotrichum gloeosporioides* in tissue of intervein however was higher than in petioles or vein tissues. It was not surprising to isolate C. *gloeosporioides*, a causal agent of anthracnose in many plants as an endophyte, because some endophytes are potential pathogens when environment factors change and induce the unknown triggering factors [36,37]. In *Wallichia caryotoides, Coletotrichum. gloeosporioides*, *Phomopsis* sp. and *Xylaria* sp. 3 had higher frequency in petioles than in vein and intervein tissues. Mycelia sterila, *Xylaria* sp. 2, 4 and 9 in vein tissues had a higher frequency in vein tissues than in petiole and intervein tissues. Some fungi were found exclusively in petioles vein or intervein tissues (Table 3, 4). This agrees with the study of Photita *et al.* [38] who found that some endophytic fungi have an affinity for different tissue types. Previous studies have indicated that endophytic fungi may exhibit tissue specificity [16,18,29,39,40]. Some of the endophytic fungi which infected *C. kerrianus* and *W. caryotoides* may be transmitted through the seed or may be systemic fungi, especially those fungi found with high frequency in every tissue type [18].

Table 2. Intensity of endophytes from petiole segments, leaf vein and intervein tissues from *Calamus kerrianus* and *Wallichia caryotoides.*

	Wet season		Dry season		Hot season	
	HKM	**MPG**	**HKM**	**MPG**	**HKM**	**MPG**
Calamus kerrianus						
Petiole	1.60	1.18	1.04	1.14	0.56	1.12
Vein	0.89	2.40	0.94	0.72	0.26	0.00
Intervein	0.98	1.34	0.76	0.50	0.04	0.08
Average	1.13	1.64	0.91	0.78	0.28	0.40
Wallichia caryotoides						
Petiole	1.06	1.26	1.12	0.68	0.68	0.48
Vein	1.34	1.96	0.56	1.12	0.00	0.34
Intervein	1.16	0.80	0.52	1.12	0.16	0.04
Average	1.19	1.34	0.73	0.98	0.28	0.28

HKM = Huay Kog Maa Site, MPG = Medicinal Plant Garden Site

Table 3. Relative frequency (%) of fungal endophytes isolated from different tissue types of *Calamus kerrianus* and *Wallichia caryotoides*.

Fungal taxa	*Calamus kerrianus*			*Wallichia caryotoides*		
	Petiole	Vein	Intervein	Petiole	Vein	Intervein
Colletotrichum gloeosporioides	0.44	1.27	1.56	1.17	1.07	0.49
Sterilia mycelia	3.85	3.61	2.54	1.85	4.00	3.02
Phomopsis sp.	1.80	0.98	0.15	6.54	1.37	0.68
Xylaria sp. 2	6.68	5.12	3.17	4.24	7.12	4.59
Xylaria sp. 3	4.49	2.93	1.61	3.32	3.12	2.24
Xylaria sp. 4	2.05	0.89	0.78	1.56	2.05	0.88
Xylaria sp. 9	2.05	0.30	0.78	0.49	2.83	1.27
Total	**21.36**	**15.10**	**10.59**	**19.17**	**21.56**	**13.17**

Table 4. Relative frequency (%) of fungal endophytes recovered from *Calamus kerrianus* and *Wallichia caryotoides* from two sites.

Fungal taxa	*Calamus kerrianus*		*Wallichia caryotoides*	
	Huay Kog Maa	Medicinal Plant Garden	Huay Kog Maa	Medicinal Plant Garden
Colletotrichum gloeosporioides	3.22	0.05	2.63	0.02
Sterilia mycelia	4.98	5.02	7.40	1.46
Phomopsis sp.	2.49	0.34	6.73	1.85
Xylaria sp. 2	4.88	10.15	2.20	13.76
Xylaria sp. 3	2.73	6.30	2.24	6.44
Xylaria sp. 4	2.44	1.46	0.78	3.71
Xylaria sp. 9	2.15	0.98	0.98	3.61
Total	**22.89**	**24.30**	**22.96**	**30.85**

3.3 Site Effect

Both study sites have similar tree species but they differ in canopy cover and elevation. There were 20 taxa of endophytic fungi recovered from Huay Kog Maa and 14 taxa at Medicinal Plant Garden. There were no significant overall differences of isolation prevalence and intensity when the Kruskal-Wallis procedure was applied, some species differed (Table 4). The frequency of *C. gloeosporioides* in both *C. kerrianus* and *W. caryotoides* was high in Huay Kog Maa, while only a few isolates were found in Medicinal Plant Garden. The frequency of *Xylaria* sp. 2 and *Xylaria* sp. 3 at Medicinal Plant Garden were higher than at Huay Kog Maa. The results were similar with the study of Photita *et al.* [38] who reported that the common fungal endophytes isolated from banana (*Musa accuminata*) samples collected from five different sites in Chiang Mai varies between sites.

3.4 Seasonal Effect

In *Calamus kerrianus*, the intensity from petioles were higher than that from vein and intervein at both sites, and in all seasons except that higher intensity was recorded on vein tissues than on petioles and intervein tissues during the wet season at Medical Plant Garden. The intensity of endophytic fungi from petioles of *W. caryotoides* was higher than those from vein and intervein tissues. The intensity of endophytes from *W. caryotoides* collected in Medicinal Plant Garden during the wet and dry seasons, was higher in vein and intervein than in the petioles. In the hot season the intensity in petioles was higher than from vein and intervein tissues (Table 2).

Seasonality and collecting site may not have affected the isolatuion prevalence and intensity in this study as it did in the study of palms by Fröhlich *et al.* [18]. They reported that the intensities for the petiole and vein tissues of *Licuala ramsayi* and *Licuala* sp. were higher than those for vein tissue in all four sites in Australia and Brunei. Isolation prevalence and intensity of endophytic fungi during wet and dry seasons were in general higher than those in hot dry season (Tables 1, 2). In a similar study of endophytic fungi in *Amomum siamense* from Huay Kog Maa and Medicinal Plant Garden [27], isolation prevalence in both wet and dry season were not different, perhaps because the study sites had high humidity, temperature and rainfall through out the year.

In each season, climatic conditions, especially temperature and moisture content at the study sites differsed. Isolation prevalence and intensity were similar in the wet and dry seasons, because at the study sites, there is still moisture in the dry season, although it is lower than in the wet season. In the hot season, the ambient humidity is low, and this affects the isolation prevalence and intensity. In the hot season isolation prevalence and intensity were lower compared with the other two seasons. However, there was no significant difference of either isolation prevalence or intensity of the two species of palm among the three seasons when the Kruskal-Wallis test was performed.

There are some fungi which had a high frequency of isolation (Table 5). In the both palms, *C. gloeosporioides*, *Xylaria* sp 2, 3, 4 and 9 (Figure 2) had high frequency in wet season while *Phomopsis* sp. (Figure 2) had a high frequency in dry season.

Table 5. Relative frequency (%) of some fungal endophytes recovered from *Calamus kerrianus* and *Wallichia caryotoides* in three seasons.

Fungal taxa	*Calamus kerrianus*			*Wallichia caryotoides*		
	Wet	**Dry**	**Hot**	**Wet**	**Dry**	**Hot**
Colletotrichum gloeosporioides	1.37	1.85	0.05	1.66	0.78	0.29
Sterilia mycelia	8.00	1.56	0.44	6.92	1.56	0.39
Phomopsis sp.	1.17	0.83	0.83	2.15	5.26	1.17
Xylaria sp. 2	6.54	7.17	1.32	10.63	4.10	1.22
Xylaria sp. 3	4.56	4.20	0.24	4.88	3.71	0.10
Xylaria sp. 4	2.73	0.98	0.20	2.73	1.27	0.40
Xylaria sp. 9	2.63	0.20	0.29	4.29	0.39	0.00
Total	**27.00**	**16.79**	**3.37**	**33.26**	**17.07**	**3.57**

Figure 1. Petioles and leaves of healthy palms. a. *Calamus kerrianus*, b. *Wallichia caryotoides.*

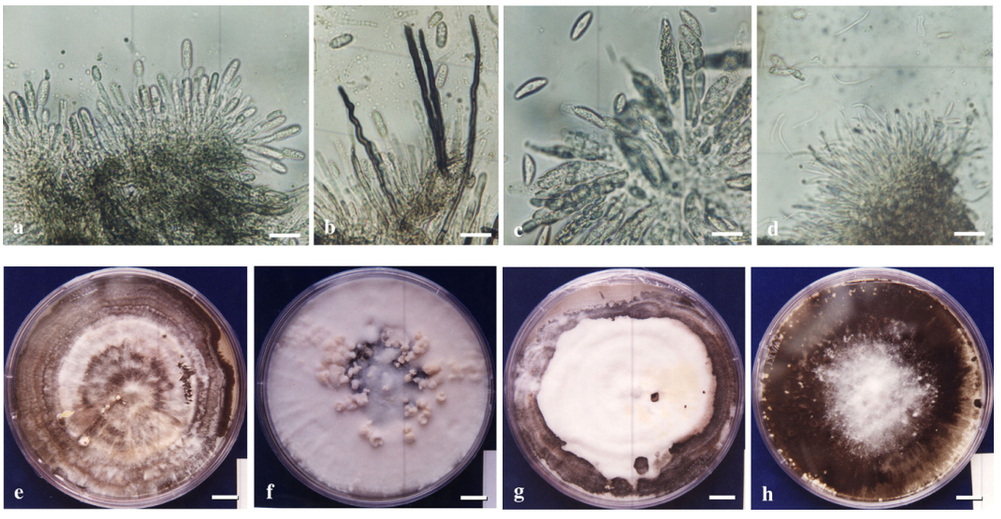

Figure 2. Most occurrences of fungal taxa isolated from *Calamus kerrianus* and *Wallichia caryotoides.* a-c. *Glomerella cingulata*, a. Conidiophores baring conidia of anamorphic *Colletotrichum gloeosporioides*, b. Dark setae of anamorphic *C. gloeosporioides*, c. asci and ascospores, d. α- and β-conidia of *Phomopsis* sp., e-h. Colonies of *Xylaria* spp. on corn meal agar, e. *Xylaria* sp. 2, f. *Xylaria* sp. 3, g. *Xylaria* sp. 4, h. *Xylaria* sp. 9. Scale bars: a-d = 10 μm, e-f = 1 cm.

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