Diversity of fungal endophytes in medicinal plants of Courtallam hills, Western Ghats, India

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A total of 3634 endophytic fungal isolates were recovered from 4800 leaf, stem and bark segments of 10 medicinal plants growing in Courtallam hills, Western Ghats, south India, during monsoon, winter and summer seasons. These isolates belonged to coelomycetes (26.35%), hyphomycetes (21.76%), Xylariaceae (0.6%) and mycelia sterilia (3.55%). Colonization frequency of endophytic fungi varied significantly between seasons. The fungal community from leaves was most diverse followed by stem and bark tissues. *Alternaria alternata*, *Camarosporium palliatum*, *Colletotrichum gloeosporioides*, *Curvularia lunata* and *Phoma terrestris* were the most frequently isolated species. *Colletotrichum gloeosporioides*, *Alternaria alternata*, *Phyllosticta* sp. 1, *Curvularia lunata* and sterile forms were isolated more frequently from the leaf than from stem and bark tissues. *Phyllosticta* sp. 1 and *Phyllosticta* sp. 2, were isolated only from leaf tissues. *Phoma terrestris* was the predominant isolate from stems while *Pestalotiopsis versicola* was dominant in bark tissues. *Xylaria* sp. was isolated only from stem tissues of *Feronia elephantum.*

Key words – Diversity – Endophytic fungi – Medicinal plants – Season

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Introduction

Endophytes are bacteria, fungi, and actionmycetes that colonize living internal tissues of plants without causing any immediate, overt negative effects (Bacon & White 2000, Botella et al. 2010, Sakalidis et al. 2011). Endophytes have been reported from all major groups of plants including algae (Hawksworth 1988, Zuccaro et al. 2008, Suryanarayanan et al. 2010), lichens (Petrini et al. 1990, Suryanarayanan et al. 2005), mosses (Petrini 1986, Schulz et al. 1993), other bryophytes (Davis et al. 2003), ferns (Petrini 1986, Petrini et al. 1992), conifers (Carroll et al. 1977, Carroll & Carroll 1978, Petrini & Muller 1979, Petrini & Carroll 1981, Petrini 1986, Giordano et al. 2009) and angiosperms (Taylor et al. 1999, Arnold et al. 2000). Endophytes occur in wide range of habitats, such as coastal mangroves (Kumaresan & Suryanarayanan 2001), temperate evergreen forests (Espinosa-Garcia & Langenheim 1990), xeric regions (Suryanarayanan et al. 2003) and tropical forests (Arnold et al. 2000). The composition of the fungal community usually differs between host species (Okane et al. 1998, Arnold 2007, Saikkonen 2007), among geographically separate individuals of the same host species (Rollinger & Langenheim 1993, Fisher et al. 1994, Collado et al. 1999), and also differ within the various tissues or organs of a host plant (Sahashi et al. 2000, Ragazzi et al. 2003, Kumar & Hyde 2004, Santamaria & Diez 2005). Variation in the diversity of fungi may

be associated with location, climate and leaf age (Petrini 1991, Asai et al. 1998). Sampling and characterizing fungal endophyte diversity is an emerging challenge, which leads to the discovery of new species, novel compounds and a better understanding of their role in ecosystems (Arnold & Lutzoni 2007, Saikkonen 2007, Rodriguez et al. 2009). The prime objective of the present study was to explore the diversity and distribution of fungal endophytes in ten medicinal plant species during various seasons in a tropical evergreen forest of Courtallam, Western Ghats, India and to describe the frequency with which endophytic fungi were recovered from leaves, stem and bark tissues.

Materials and methods

Collection of plant sample

The survey was made for one year at Courtallam hills, from healthy leaf, stem and bark samples of ten medicinal plant species (Table 1). The plant materials were collected in sterile polythene bags and used for the isolation of endophytic fungi.

Isolation of endophytic fungi

Asymptomatic healthy plant materials were thoroughly washed in running tap water then surface sterilized by a modified method of Raviraja (2005). The selected leaf, stem and bark segments were immersed in 95% ethanol for 30 s, 4% sodium hypochlorite solution for 60 s and 95% ethanol for 30 s followed by rinsing with sterile distilled water three times for 10 s and allowed to surface dry under sterile conditions. After drying, each leaf segment was cut into approximately 0.5 cm squares and placed on Petri dishes containing potato dextrose agar medium (PDA) supplemented with streptomycin (250µg/L) to suppress bacterial growth. Petri dishes were sealed with cling film and incubated at 30°C in a light chamber for up to one week. They were monitored every day for growth of endophytic fungal colonies. Fungi growing out from the samples were subsequently transferred onto fresh PDA plates. The procedure of transferring to fresh PDA plates was carried out several times in order to isolate pure colonies. The endophytic fungal isolates were identified at Mycological unit, TBGRI, Tiruvananthapuram and CAS in botany, University of Madras, Chennai, India.

Statistical analysis

Colonization Frequency (CF%)

The colonization frequency (CF%) of a single endophytic fungal species in the leaf, stem and bark segments was calculated by using the following formula (Hata & Futai 1995):

$$CF\% = \frac{\text{Number of segments colonized by an endophytic fungal species}}{\text{Total number of segments}} \times 100$$

Relative percentage occurrence (RPO%) of each group of fungi

Relative percentage of occurrence (RPO%) of different group of fungi viz., coelomycetes, hyphomycetes, xylariaceous and other fungi was calculated using the following formula:

$$RPO\% = \frac{\text{Density of colonization of one fungal group}}{\text{Total density of colonization of all fungal groups}} \times 100$$

Species diversity

Shannon diversity index (H'), Shannon evenness index (J') and Simpson diversity index (1/D) were used for the evaluation of fungal species richness (Brower 2004).

Results

A total of 3634 endophytic fungal isolates were recovered from leaf, stem and bark segments incubated from ten medicinal plants during southwest monsoon, northeast monsoon, winter and summer seasons. These isolates belonged to coelomycetes (26.35%), hypomycetes (21.76%), xylariaceous (0.6%) and mycelia sterilia (3.55%). Colonization of endophytic fungi ranged from 41.0–67.0%. Highest colonization of 67.0% and 61.3% were observed in *Feronia elephantum* and *Madhuca longifolia*, respectively. Total colonization frequency of 73.0%, 85.0%, 81.0% and 62.0%

Table 1 Medicinal plant from which endophytic fungi were isolated.

Plant	Habit	Family	Medicinal uses
Cassia auriculata L.	Shrub	Caesalpiniaceae	Skin disorders
Feronia elephantum Corrêa.	Tree	Rutaceae	Asthma
Justicia gendarussa Burm. f.	Shrub	Acanthaceae	Fever
Madhuca longifolia J.F. Macbr.	Tree	Sapotaceae	Diabetes
Morinda tinctoria Roxb.	Small tree	Rubiaceae	Gastric ulcer
Murraya koenigii L.	Small tree	Rutaceae	Blood purifier
Phyllanthus emblica L.	Tree	Euphorbiaceae	Antioxidant
Pongamia glabra Vent.	Tree	Fabaceae	Bleeding piles
Sesbania aegyptiaca Pers.	Shrub	Fabaceae	Tuberculosis
Thespesia populnea Cav.	Tree	Malvaceae	Anti-inflammatory

were found during southwest monsoon, northeast monsoon, winter and summer seasons respectively. There was a significant difference in the colonization frequency (%) of endophytic fungi in various seasons. *Alternaria alternata, Camarosporium palliatum, Colletotrichum gloeosporioides, Curvularia lunata* and *Phoma terrestris* were the dominant species. *Colletotrichum gloeosporioides, Phoma terrestris* and *Phyllosticta* sp1, were dominant in northeast monsoon and winter seasons. *Alternaria alternata, A. brassicola, Aureobasidium pullulans, Fusarium oxysporum, Phoma trachelii* and *Scopulariopsis brevicaulis* were obtained in the southwest monsoon season. *Alternaria solani, Aspergillus* sp, *Colletotrichum accutatum, C. gloeosporioides, C. graminicola, Curvularia brachyspora* and *Xylaria* sp. were isolated most frequently in the summer season. Colonization frequency was highest in *Morinda tinctoria* (83.3%), *Pongamia glabra* (93.3%), *Pongamia glabra* (87.0%) and *Morinda tinctoria* (76.0%) during southwest monsoon, northeast monsoon, winter and summer seasons, respectively (Table 2).

The Shannon diversity index (H') was highest in *Madhuca longifolia* (H'= 2.60) and lowest in *Sesbania aegyptiaca*. (H'= 2.32). The Shannon evenness index (J') was highest in *Madhuca longifolia* (J'= 2.27) and *Phyllanthus emblica* (J'= 2.27), and lowest in *Sesbania aegyptiaca* (J'= 2.15). Simpson diversity index revealed the highest abundance of endophytic fungi in *Feronia elephantum* (1/D = 13.65) with 18 species and least abundance in *Sesbania aegyptiaca* (1/D = 9.22) with 13 species (Table 2).

Generally the leaf samples showed more diversity of endophytic fungi than the stem and bark samples. The number of isolates recovered from leaf, stem and bark samples were 1761, 1140 and 733, respectively (Table 3). Species of *Colletotrichum gloeosporioides, Alternaria alternata, Curvularia lunata, Pestalotiopsis versicola, Phoma terrestris* and mycelia sterilia were observed as the dominant in leaf, stem and bark samples. *Xylaria* sp. was found only in the stem tissues of *Feronia elephantum*. Mycelia sterilia was a large group of fungi that failed to sporulate and ubiquitous in all the plant endophytic isolations (Table 3).

The study revealed that *Colletotrichum* sp, had the highest relative frequency of 22.67% whereas *Alternaria* sp, *Phoma* sp, *Curvularia* sp, *Aureobasidium* sp, *Truncatella* sp. and mycelia sterilia had relative frequencies of 13.95%, 9.36%, 8.59%, 5.04%, 4.32%, and 3.36, respectively. *Xylaria* sp. had the lowest relative frequency of 0.17% (Fig 1).

Discussion

As tropical and subtropical climates harbour most of the worlds plant diversity, so endophytic diversity in this climatic zone is also higher as almost all vascular plant species examined to date are found to possess endophytic bacteria and fungi (Firakova et al. 2007). Most undescribed fungal diversity lies within the tropical plant associated fungi, yet the diversity and ecological role of endophytes in tropical angiosperms are almost entirely unexplored (Hawksworth 1993, Rodrigues & Petrini 1997). The colonization frequency of endophytes in this study was within the range of many host plants studied in the tropics (Fröhlich et al. 2000, Photita et al. 2001, Suryanarayanan et al. 2003). *Alternaria alternata, Camarosporium palliatum, Colletotrichum gloeosporioides, Curvularia lunata* and *Phoma terrestris* which were dominant in this

Table 2 Colonization frequency (%) of endophytic fungal groups, dominant genus and total number of species encountered, together with Shannon, Evenness and Simpson diversity indices for different medicinal plants.

Host plant	Fungal groups (%)					Colonization frequency (%)								
	C	H	X	St	Total	SM	NM	W	S	No. of species	Dominant genus	Sh (H')	Ev (J')	Si (1/D)
Cassia auriculata	32.3	19.1	-	2.3	54.0	77.0	83.3	80.0	60.0	15	*Colletotrichum*	2.43	2.18	11.01
Feronia elephantum	32.0	27.0	6	2.0	67.0	66.0	82.0	82.0	59.3	18	*Curvularia*	2.59	2.20	13.65
Justicia gendarassa	30.0	26.0	-	4.0	60.0	68.3	88.0	80.0	57.5	15	*Alternaria*	2.46	2.21	11.26
Madhuca longifolia	29.0	27.3	-	5.0	61.3	75.0	88.3	82.0	63.3	17	*Truncatella*	2.60	2.27	13.56
Morinda tinctoria	27.0	22.2	-	3.3	53.0	83.3	89.0	86.0	76.0	15	*Colletotrichum*	2.50	2.24	11.89
Muraya koenigii	27.3	18.0	-	1.0	46.0	82.2	90.0	83.3	68.0	14	*Curvularia*	2.44	2.19	11.12
Phyllanthus emblica	24.0	19.o	-	3.0	46.0	71.1	83.3	80.0	63.3	16	*Colletotrichum*	2.53	2.27	12.69
Pongamia glabra	19.0	27.0	-	5.3	51.3	73.3	93.3	87.0	59.0	15	*Colletotrichum*	2.40	2.22	10.95
Sesbania aegyptiaca	20.0	18.0	-	3.3	41.0	57.0	69.0	70.0	53.3	13	*Phoma*	2.32	2.15	9.22
Thespesia populniea	23.4	14.2	-	7.0	44.2	72.2	82.2	76.0	57.0	17	*Colletotrichum*	2.52	2.26	12.49
Mean	26.35	21.76	0.6	3.55	52.3	73.0	85.0	81.0	62.0	-	-	-	-	-

C = Coelomycetes, H = Hyphomycetes, X = Xylariaceous, St = Myecila sterile, SM = Southwest monsoon, NM = Northeast monsoon, W = Winter, S = Summer, Sh = Shannon, Ev = Eveness, Si = Simpson.

Table 3 Number of endophytic fungal isolates recovered from various tissues of medicinal plants.

Fungi	Tissue			
	Leaf	**Stem**	**Bark**	**Total**
Xylaria sp.	0	6	0	6
Alternaria sp.	258	146	103	507
Aspergillus sp.	49	35	34	118
Aureobasidium sp.	92	60	31	183
Bipolaris sp.	45	24	9	78
Cladosporium sp.	24	12	16	52
Curvularia sp.	137	104	71	312
Fusarium sp.	57	38	13	108
Nigrospora sphaerica	66	53	31	150
Scopulariopsis sp.	66	52	24	142
Botryodiplodia sp.	54	31	29	114
Camarosporium sp.	58	41	21	120
Colletotrichum sp.	438	242	144	824
Myrothecium sp.	11	9	9	29
Pestalotiopsis sp.	58	33	72	163
Phoma sp.	109	170	61	340
Phyllosticta sp.	109	0	0	109
Truncatella sp.	82	47	28	157
Mycelia sterilia	48	37	37	122

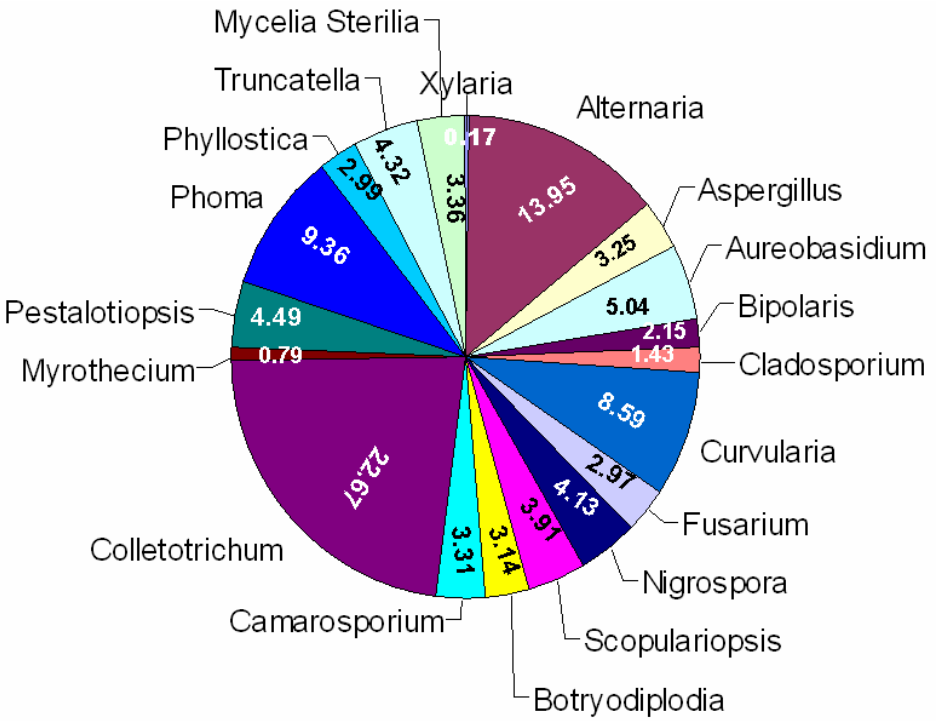

Fig. 1 – Relative frequencies of isolation of endophytic fungal genera from ten medicinal plants.

study, have been reported as endophytes in a wide host range in the tropics (Azevedo et al. 2000, Suryanarayanan et al. 2002, Photita et al. 2005). In this study, Coelomycetes were found to be dominant and two *Truncatella* sp. and *Myrothecium* sp. are reported for the first time as endophytic fungi in a tropical forest.

Higher colonization frequency and greater diversity of endophytes were observed during the monsoon and winter seasons than in summer. The greater number of fungal isolates suggested that colonization by endophytes is correlated with climatic factors, such as rainfall and atmospheric humidity, which might influence the occurrence of some endophytic species (Wilson & Carroll 1994). Earlier studies also recovered fewer isolates during the dry season and suggested this could be related

to the effects of water (Rodrigues 1994). It is known that under water deficit some plants may accumulate non-structural carbohydrates. This accumulation generally leads to a buildup of carbon-based defenses such as tannins, making the plant less susceptible to fungal endophyte colonization during the dry season (Rodrigues 1994, Suryanarayanan et al. 1998).

Alternaria alternata, Botryodiplodia theobromae, Camarosporium palliatum, Colletotrichum gloeosporioides, C. graminicola, Curvularia lunata, Fusarium oxysporum, Phyllosticta sp1, *Myrothecium* sp, *Truncatella* sp. and sterile forms were isolated more frequently from leaf than from stem and bark tissues. *Phoma terrestris, P. trachelii* and *Xylaria* sp. were more prevalent isolates from stem tissues, but *Pestalotiopsis versicola* was dominant in bark tissues. Similarly, Huang et al. (2008) reported that some endophytic fungi more frequently colonized leaves than stems. More endophytic isolates of *Colletotrichum gloeosporioides* were obtained from the leaves than from the stems. The difference in endophyte assemblages from various tissues would suggest that some fungal endophytes have an affinity for different tissue types, and this might be a reflection of their capacity for utilizing or surviving within specific substrates (Petrini & Carroll 1981, Rodrigues 1994, Photita et al. 2001). Mycelia sterilia were frequently found in leaf, stem and bark segments of the plants. Mycelia sterilia have been often isolated as leaf endophytes from many host plants (Suryanarayanan et al. 1998, Rajagopal et al. 2000, Huang et al. 2008).

Tropical endophytes constitute a diverse but poorly known group. Further study of endophyte ecology in natural systems promises to elucidate both potential applications of endophytic fungi for human use and ecological roles of these ubiquitous associates of healthy plant tissues. This study shows that the endophytic assemblages of medicinal plants of Courtallam hills are high in diversity and abundance.

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