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Diversity of fungal endophytes in shrubby medicinal plants of Malnad region, Western Ghats, Southern India

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ABSTRACT

A total of 6125 fungal endophytes were isolated from 9000 leaf segments of 15 medicinal shrubs growing in Malnad region of Western Ghats, Southern India, during winter, monsoon, and summer seasons. These fungal isolates belonged to Ascomycota (8.6 %), Coelomycetes (26.0 %), Hyphomycetes (28.0 %), Mucoromycotina (0.3 %) and sterile forms (4.9 %). *Alternaria*, *Chaetomium*, *Fusarium*, *Colletotrichum*, *Cladosporium*, *Penicillium*, *Phyllosticta* and *Xylaria* were the most frequently isolated. Significantly more isolates were obtained during the winter season than monsoon and summer seasons.

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Introduction

Foliar fungal endophytes are a fundamental but frequently overlooked aspect of plant biology (Arnold & Lutzoni 2007). They are ubiquitous and have been found in all plant species examined to date (Stone *et al.* 2000). Endophyte host interactions represent a continuum from strong antagonism to obligate mutualism (Saikkonnen *et al.* 1998; Clay & Schardl 2002). Endophytes may also act as pathogens and saprotrophs, in some cases attacking or decaying hosts with which they do not form endophytic associations (Promputtha *et al.* in press). In mutualistic associations, infected plants benefit by, for example, exhibiting increased resistance to herbivore grazing through the production of various alkaloids (Owen & Hundley 2004), improved growth and competitive ability by increasing the mineral uptake potential, plant phenotypic traits, temperature and drought tolerance, leaf chemistry, tolerance of heavy metals in soils, propensity for vegetative reproduction (Malinowski *et al.* 2000; Redman *et al.* 2002) and defence against microbial pathogens (Arnold *et al.* 2003; Rubini *et al.* 2005). Further, fungi present in the healthy tissues of plants can promote the invasion of host plant communities with greater species diversity (Rudgers *et al.* 2005) and alter the nutrient cycle in individual plants and in ecosystems (Garcia & Langenheim 1990; Lodge *et al.* 1996).

In addition, fungal endophytes have been recognized as a repository of novel compounds of immense value in agriculture, industry and medicine (Tan & Zou 2001; Strobel & Daisy 2003; Kumar *et al.* 2004; Kumar *et al.* 2005). Hence, there are major efforts to isolate and characterize endophytes from plants that have an ethnobotanical history (i.e. use by indigenous peoples) that is related to the specific uses or applications. Ongoing global efforts to discover novel compounds from endophytic fungi isolated from medicinal plants are

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yielding good results (Liu et al. 2004; Li et al. 2005; Phongpaichit et al. 2006; Zhou et al. 2007). People in the Indian subcontinent have a long history of using medicinal plants to cure various diseases. Medicinal plants of Western Ghats of India (one of the hot spots of global biodiversity) are reported to have a diverse community of endophytic fungi (Raviraja 2005; Krishnamurthy et al. 2008). Few studies on the endophytic fungi of these plants have been conducted. The present study was undertaken to investigate the diversity of endophytic fungi and their seasonal colonization pattern in medicinal shrub species commonly used in the Malnad region, Western Ghats of Karnataka, Southern India.

Materials and methods

Sample collection and isolation of endophytes

Apparently healthy leaf samples from 15 plant species (Table 1) were collected, brought in sterile polythene bags to the laboratory and processed within 24 h of collection. Surface sterilization of samples was done by cleaning leaves under running tap water and cutting them into 1 cm segments followed by stepwise washing with 70 % ethanol for 2 min, sodium hypochlorite solution for 5 min and 70 % ethanol for 30 s followed by two rinses in sterile distilled water, then allowed to surface dry under sterile conditions (Arnold et al. 2000). Leaf segments were placed on 9 cm Petri plates containing potato dextrose agar (PDA, Hi Media Laboratories, Mumbai, India) medium amended with streptomycin (250 mg L^{-1}) to suppress bacterial growth. The efficacy of surface sterilization was confirmed by pressing the sterilized leaf segments on to the surface of PDA medium. The absence of growth of any fungi on the medium confirmed that the surface sterilization procedure was effective (Schulz et al. 1993). Petri plates were incubated at 28 ± 1 °C with a 12 h photoperiod, and sporulation was induced by incubation in a light chamber under near UV light for 1–12 d. Fungi growing out from the leaf segments were subsequently transferred onto fresh PDA plates. Pure cultures were spread on fresh PDA slants. Endophytic fungal species were identified on the basis of cultural characteristics and morphology of fruit bodies and spores. Cultures that failed to sporulate were recorded as sterile form. Two collections were made in each season. From each medicinal plant 200 segments were randomly selected from leaves of two individuals/season. All isolates are maintained in Culture Collection Centre of Department of Applied Botany, Kuvempu University, Shankaraghatta, India.

Data analysis

Frequency of colonization by endophytic fungi was determined as the total number of segments yielding $\geq$1 isolate in a host sample divided by total number of segments incubated in that sample $\times$ 100. Frequency of colonization by individual taxa was calculated similarly. Significance of differences in the frequency of colonization among the host plants was determined by Kruskal Wallis method (Gibbons 1976). Differences between winter, monsoon and summer seasons were tested by ANOVA. Shannon diversity index (H'), Shannon evenness index (J') and Simpson diversity index ($1/D$) were used for the evaluation of fungal species richness (Zar 2004). Jaccard's similarity coefficient (JI) was used to describe the taxonomic affinity of endophytic mycobiota among host samples (Arnold et al. 2000).

Results

A total of 6125 fungal isolates were recovered from 9000 leaf segments incubated from 15 medicinal shrubs during winter, monsoon, and summer seasons. These isolates belonged to Ascomycota (8.6 %), Coelomycetes (26.0 %), Hyphomycetes (28.0 %), Mucoromycotina (0.3 %) and sterile forms (4.9 %) (Table 2). *Alternaria*, *Chaetomium*, *Fusarium*, *Colletotrichum*, *Cladosporium*, *Penicillium*, *Phyllosticta* and *Xylaria* were the most frequently isolated genera. Colonization did not differ significantly among medicinal shrub species but ranged from 39.1 % in *Hibiscus rosa sinensis* to a maximum of 89.6 % in *Carissa carandus*. Colonization (%) differed significantly between the

Table 1 – Plants from which endophytic fungal isolations were attempted

Host plant	Family	Collection site	Medicinal uses
Adathoda vasica	Acanthaceae	Thirthahalli	Bronchitis, fever, cough
Calotropis gigantean	Asclepidiaceae	Shimoga	Leprosy, ulcer, asthma
Carissa carandus	Apocyanaceae	Shankaraghatta	Antifungal
Cassia alata	Fabaceae	Shankaraghatta	Antibacterial
Citrus medica	Rutaceae	Thirthahalli	Skin disorders
Datura metel	Solanaceae	Sagar	Antifungal
Ervatomia coronaria	Apocyanaceae	Kumsi	Eye diseases
Hibiscus rosa sinensis	Malvaceae	Sagar	Gastro intestinal disorders
Ixora coccinea	Rubiaceae	Thirthahalli	Dysentery
Jatropha curcus	Euphorbiaceae	Lakkavalli	Eczema
Lantana camara	Verbenaceae	Lakkavalli	Blood clotting
Nerium indicum	Apocyanaceae	Kumsi	Cardio tonic, skin diseases
Punica granatum	Puniaceae	Koppa	Dysentery
Toddalia asiatica	Rutaceae	Koppa	Gastro intestinal disorders
Vitex nigundo	Verbenaceae	Sagar	Antibacterial

Table 2 – Colonization frequency (%) of fungal classes, dominant genus and total number of species encountered, with Shannon, Eveness and Simpson diversity indices for different medicinal shrubs in Malnad region, Karnataka

Host plant	Fungal classes (%)					Total	Colonization frequency (%)			Dominant genus	No. of species	Shannon (H')	Eveness (J')	Simpson ($1/D$)
	Asco	Coelo	Hypho	Muco	Sterile		Monsoon	Winter	Summer					
Adathoda vasica	3.6	27	35.5	–	–	66.1	66	78	54.5	*Phyllosticta*	14	0.99	0.86	7.14
Calotropis gigantean	11	35.6	23.6	1.8	4.6	76.1	66.5	94	68	*Phyllosticta*	10	0.92	0.92	7.69
Carissa carandus	–	30.6	59	–	–	89.6	92	99	78	*Phyllosticta*	13	0.94	0.84	7.14
Cassia alata	3	24.1	31.3	2.5	–	61	60.5	84	38.5	*Phomopsis*	11	0.91	0.85	6.66
Citrus medica	24.5	25.8	28	–	1.3	79.6	95	99.5	44.5	*Glomerella*	17	1.08	0.87	10
Datura metel	14.5	30.8	29.6	–	9.6	84.6	71	110	73	*Phomopsis*	13	0.95	0.85	6.66
Ervatomia coronaria	3.6	40	27	–	–	70.6	73	83.5	55.5	*Colletotrichum*	12	0.84	0.78	4.76
Hibiscus rosa sinensis	–	5.5	23.5	–	10.1	39.1	33.5	64	20	*Curvularia*	9	0.9	0.94	7.69
Ixora coccinea	–	49.3	25.5	0.6	3	78.5	99	96.5	40	*Phyllosticta*	13	0.85	0.74	4.76
Jatropha curcus	17.1	3.1	21.3	–	16.8	58.5	60	73	42.5	*Leptosphaeria*	11	0.89	0.89	6.25
Lantana camara	10.3	24.3	31.3	–	0.8	66.8	72	75.5	53	*Colletotrichum*	13	0.87	0.78	5.55
Nerium indicum	1.8	18	31.5	–	2.5	53.8	58.5	65	38	*Phyllosticta*	12	0.97	0.9	8.33
Punica granatum	27.5	19	11.33	–	7	66.6	70.5	93.5	36	*Phyllosticta*	10	0.89	0.89	6.66
Toddalia asiatica	11.5	30	17.5	–	2.8	61.8	59.5	67.5	58.5	*Colletotrichum*	16	1.05	0.87	9.09
Vitex nigundo	–	27	24.8	–	15.6	67.5	80	86	36.5	*Phyllosticta*	14	1.02	0.89	9.09

Asco = Ascomycota, Coelo = Coelomycetes, Hypho = Hyphomycetes, and Muco = Mucoromycotina.

seasons (PF = 3.86) with 46.6 %, 55.7 % and 37.9 % colonization in monsoon, summer and winter seasons, respectively. It was highest in *Ixora coccinia* (99.0 %) and lowest in *H. rosa sinensis* (33.5 %) during the monsoon season; highest in *Datura metel* (110.0 %) and lowest in *H. rosa sinensis* (64.0 %) during the winter season; and highest in *C. carandus* (78.0 %) and lowest in *H. rosa sinensis* (20.0 %) during the summer season (Table 2). Shannon diversity index (H') was highest in *Citrus medica* ($H' = 1.08$) and lowest in *Ervatomia coronaria* ($H' = 0.84$). The Shannon evenness index was highest in *H. rosa sinensis* ($J' = 0.94$) and lowest in *I. coccinia* ($J' = 0.74$). Simpson's diversity index revealed the highest abundance in *C. medica* ($1/D = 10.0$), with a maximum of 17 species, and least abundance in *E. divaricata* ($1/D = 4.76$) and *Ixora coccinea* ($1/D = 4.76$) with 12 and 13 species, respectively (Table 2).

Species in the genera *Aschersonia*, *Botryosphaeria*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Myrothecium*, *Penicillium*, *Phomopsis* and *Phyllosticta* were isolated most frequently in the monsoon season; *Alternaria*, *Aschersonia*, *Aspergillus*, *Aureobasidium*, *Chaetomium*, *Colletotrichum*, *Curvularia*, *Glomerella*, *Leptosphaeria*, *Myrothecium*, *Pestalotiopsis*, *Phomopsis* and *Phyllosticta* in the winter season; and *Cladosporium*, *Penicillium*, *Verticillium* and *Xylaria* in the summer season. Sterile forms were consistently isolated frequently from *D. metel* (9.6 %), *H. rosa sinensis* (10.1 %), *Jatropha. curcas* (16.8 %), *Punica granatum* (7.0 %) and *Vitex nigundo* (15.6 %). Colonization (%) was maximum in Shimoga (76.1 %) followed by Thirthahalli (74.7 %), Shankaraghatta (72.7 %), Koppa (64.2 %), Lakkavalli (62.65 %), Kumsi (62.2 %) and least in Sagar (61.85 %). There was no significant difference in the endophytic assemblages among the sites studied.

Discussion

The colonization frequency of endophytes in this study was within the range of many host plants studied in the tropics (Frohlich *et al.* 2000; Photita *et al.* 2001; Suryanarayanan *et al.* 2003). *Colletotichum*, *Phomopsis*, *Phyllosticta*, *Pestalotiopsis*, *Cladosporium* and *Xylaria* spp., which were isolated frequently in this 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). The greater number of total isolates in winter and monsoon seasons than in the summer season suggests that colonization by endophytes is correlated with climatic factors (Wilson & Carroll 1994). These factors may determine spread and germination success of endophytic fungal spores (Schulthess & Faeth 1998). Fungi such as *Colletotrichum*, *Phomopsis* and *Pestalotiopsis* produce slimy conidia that are not forcibly released but dispersed by water, which may account for increased isolation in the wet season. In winter high humidity and moderate temperature may allow the fungal propagules to germinate successfully. The higher incidence of species in the genera *Cladosporium* and *Penicillium* isolated during the summer may be due to the ability of their spores to survive and even grow at low water potentials. Variation in the colonization frequency among the sites might be due to site specific factors. However, quantitative surveys of endophyte

colonization patterns may be sensitive to leaf size, age, methodology, and growth medium (Lodge et al. 1996; Gamboa et al. 2002). The fungi isolated in the present work are possibly the most easily selected under the culture conditions used.

Mycelia sterilia have been isolated as endophytes from a wide range of host plants (Arnold et al. 2000; Frohlich et al. 2000). In a study on the Chinese medicinal plant *Tripterygium wilfordii*, mycelia sterilia were isolated as the second most dominant taxa (23.6 %) next to Coelomycetes (35.0 %) (Kumar & Hyde 2004). Since these non-sporulating mycelia sterilia cannot be provided with taxonomic names without reproductive structures in conventional classification they are now generally categorized as ''morphotypes'' based on similar cultural characteristics (Guo et al. 2003; Promputtha et al. 2005). Molecular techniques such as ITS sequence analyses (Promputtha et al. 2005; Wang et al. 2005) and DGGE (denaturing gradient gel electrophoresis) have now been used effectively for the phylogenetic classification of morphospecies of endophytes (Jeewon & Hyde 2006), and Hambleton & Sigler (2005) described a new genus with three new taxa based on gene sequence data of sterile mycelia. A major concern in endophyte studies is that the endophytes are isolated from surface sterilized living leaves onto artificial media (Ganley & Newcombe 2006; Hyde & Soytong 2007). Many endophytes might therefore remain undetected. Use of molecular approaches directly on plant tissues might give a wider indication of endophytes present. Duong et al. (2006) obtained 14 operational taxonomic units (OTUs), using DGGE, of fungi not normally isolated by conventional methodology from living leaves of *Magnolia* (Hyde & Soytong 2007).

The role of the detected endophyte fungi through mutual interaction in antagonism against phytopathogens or insect herbivores could be speculated. In a study on endophytes of *Oryza sativa* from Malnad region, species of the genera *Chaetomium*, *Penicillium* and *Streptomyces* exhibited antifungal activity against various phytopathogens *in vitro* (Shankar et al. 2007). In a similar antimicrobial evaluation study from medicinal plants of Western Ghats of India, fermentation cultures of *Alternaria* sp., *Nigrospora oryzae* and *Papulospora* sp. showed inhibitory activity against both Gram positive and Gram negative bacteria and also against *Candida albicans* (Raviraja et al. 2006). We are currently investigating secondary metabolites from fungi isolated in the present study both to understand their ecology and to determine potential as therapeutic targets.

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