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Mycoflora associated with Aegle marmelos, a medicinal plant of the forests of Western Ghats, India

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ABSTRACT

Aegle marmelos, a common medicinal plant of the forests of Western Ghats in southern India, was screened for its phylloplane, endophyte and saprotrophic fungal diversity. Comparison of the three microhabitats was done to determine the overlap and uniqueness in species composition. Similarly, within the endophytes overlap and uniqueness in species composition of the three tissue types was calculated. Impact of collection sites and seasons on the diversity, individual fungal colonization and tissue colonization was determined. The average fungal colonization of *Aegle marmelos* under different conditions was found to be 82%. The study resulted in the documentation of 51 sporulating and non-sporulating isolates of microfungi.

Keywords: Microfungi, endophytes, phylloplane, saprotrophs, tissue colonization

INTRODUCTION

Richness in fungal diversity is attributed to the highly diverse plant-species composition and varied vegetation types of the ecosystems especially in the tropics from where majority of the fungi have been discovered (Hyde, 1997). The forest range of Western Ghats in southern India is one of the mega-biodiversity zones identified in the tropics where diverse plant, animal and microbial life co-exists. Plant-associated microhabitats such as leaf surface, internal plant tissues and plant debris harbour high diversity of fungi (Bhat, 2009). The microhabitats of several plants have been screened to document the associated fungal diversity and to understand the ecological role played by the fungi in such associations (Jacob, 2000; D'Souza, 2002).

Medicinal and aromatic plants, due to their chemical constituents, are thought to harbour only some fungi that have the ability to grow and survive in the presence of these chemicals. Besides, in certain cases, it is observed that the fungi associated with such plants are capable of producing identical compounds, possibly as a result of horizontal gene transfer. Efforts were therefore made worldwide to document fungi occurring on medicinal and aromatic plants (Alwadi & Baka, 2001; Santos & Rodrigues, 2003; Radu *et al.*, 2000; Wiyakrutta *et al.*, 2004). There are reports on the diversity and ecological role of fungi on Eucalyptus, one of the important medicinal plants (Faifer & Bertoni, 1988; Bettucci & Saravay, 1993; Lupo *et al.*, 2001).

In India, studies on fungi occurring on medicinal and aromatic plants were done by Raviraja (2005), Ganguly & Pandotra (1963), Krishnamurthy & Hemalatha (2003), Rajagopal & Suryanarayanan (2000), Mahesh *et al.*, (2005), Nalini *et al.* (2005), Tejesvi *et al.* (2006) and Gangadevi & Muthumary (2006). These workers documented the endophytic fungi associated with medicinal plants while the phylloplane or saprotrophic assemblages remained untouched. Verma *et al.* (2007) reported only the endophytes associated with different plant parts of *Aegle marmelos*.

This paper deals with the results of a study carried out on phylloplane, endophyte and saprotrophic fungi associated with *Aegle marmelos* (L.) Corr. (F: Rutaceae), one of the common medicinal plants of the Western Ghats, India.

MATERIALS AND METHODS

Two study sites, 'Site I' at Colem, Sanguem Taluka and 'Site II' at Mashem, Canacona taluka, chosen for collection of fungal samples, were forested areas of the Western Ghats in Goa State. Site I and II are distanced, as crow flies, by 44 km. The location, topography and vegetation type of both sites are given below (Table 1).

Table 1: Location, topography, soil and vegetation type of study Site I and II

Sampling site	Site I (Colem)	Site II (Mashem)
Location	15°.19.801′ N, 74°.12.486′E	14°.57.699′N 74°.03.192′E
Topography	Undulating hilly terrain, 45 km away from sea coast	Lateritic rocky plateau
Soil type	Thick humus mixed with soil	Lateritic with thin soil
Vegetation	Dense, with wild trees bushy under storey	Sparse

Aegle marmelos is a perennial tree. The plant parts investigated included fresh leaves (for phylloplane fungi); fresh leaves, stems and bark (for endophytic fungi) and dead leaves and twigs (for litter fungi). Situated in the south-west monsoon belt, the study sites have a warm and humid climate all through the year. Accordingly, the collections were grouped into two broad seasons, *viz.* 'wet' (June to October) and 'dry' (December to May). During the study period, the wet and the dry seasons had an average rainfall of 519 mm and 46 mm, percent humidity of 90 and 80 and average sunshine hours/day of 4.6 and 9.1, respectively.

About 10 g of decayed plant material was sampled out for isolation of saprotrophs, during each sampling. This included leaf or stem litter at different stages of decomposition and randomly gathered from a single plant type. For endophytic isolation, 20-30 leaves and 5-10 stem/root/bark pieces formed a sample. For analyses of leaf surface mycoflora, randomly collected 15-20 leaves formed a sample. Sample number is the number of times a defined sample is sourced. In this study, six samples of each type in each season were collected during two years of sampling. Standard isolation techniques, *viz.* moist chamber incubation (Rossman *et al.*, 1998), particle plating (Bills & Polishhook, 1994), three-step sterilization (Petrini, 1986) and leaf washing techniques (Girivasan & Suryanarayanan, 2004) were followed for isolation and maximum recovery of fungi. Malt extract agar (2%) medium was used for culturing and maintenance of the isolates. The plates were incubated at 25°C and routinely examined for growth and sporulation of the fungi. The sporulating fungi were identified using standard taxonomic keys and monographs (Ellis, 1971, 1976; Matsushima, 1971, 1975; Seifert, 2000; Sivanesan, 1984; Sutton, 1980; Barnett & Hunter, 1972; Kifler & Morelet, 2000; Ainsworth *et al.*, 1973; Carmichael *et al.*, 1980; Onions *et al.*, 1981). In addition, several extended research papers related to taxonomy of fungi were consulted (Hughes, 1978; Bhat & Kendrick, 1993; Subramanian & Bhat, 1987; Rao & de Hoog, 1986). Fungal taxonomy related websites such as those of Centraalbureau voor Schimmelcultures, Index fungorum, Mycobank, and Systemic Mycology and Microbiology Laboratory were useful in the identification of most fungi. The cultures that remained non-sporulating were grouped as morphotypes based on distinct cultural characters such as colony diameter, height and depth, presence/absence of aerial mycelium, reverse of the colony, surface texture, margin character and growth rate.

Data gathered from this study was viewed and analyzed using the observational approach (i.e. data measurements was made over a range of conditions imposed by nature such as effect of different substrates, tissue type, locations and seasons) to determine the diversity of fungi on them. Effect of location and season on diversity of fungi was studied by comparing similar samples collected in same season from different locations and similar samples collected in different seasons from same location, respectively. Diversity indices for the same were estimated for each tissue type. Percent colonization by each fungus and overall percent tissue colonization was determined using the formulae,

$$\text{Percent Colonization by each fungus} = \frac{\text{No. of segments colonized by each fungus} \times 100}{\text{Total segments plated}}$$

$$\text{Percent tissue colonization} = \frac{\text{Total segments colonized for each tissue type} \times 100}{\text{Total segments plated for that particular tissue type}}$$

Diversity indices were calculated using the PAST software (Hammer *et al.*, 2001)

RESULTS AND DISCUSSION

The study resulted in the recovery of 51 isolates of fungi belonging to 32 species in 25 identified genera. Three species in 3 genera belonged to Ascomycetes, 17 species in 12 genera to Hyphomycetes and 12 species in 10 genera to Coelomycetes. Two taxa of Coelomycetes could not be assigned to any genera in absence of relevant literature and these were designated as undetermined coelomycete (UC). Two of the non-sporulating cultures exhibiting clamp connections in their hyphae were designated as undetermined basidiomycetes (UB). The remaining, based on their cultural characters such as colony colour, texture, margin,

Table 2: Fungi on microhabitats of Aegle marmelos (single season)

List of fungi	Site 1			Site 2		
	Pf	En	Sp	Pf	En	Sp
Acremonium curvulum	–	–	–	–	+	–
Acremonium strictum	–	–	+	+	–	–
Aspergillus fumigatus	+	+	+	–	+	–
Aspergillus niger	–	+	–	+	+	–
Aspergillus sclerotiorum	–	–	–	–	–	+
Cladosporium cladosporioides	–	–	+	+	+	+
Coleophoma empetri	–	+	–	–	–	–
Colletotrichum gloeosporioides	–	+	–	–	–	–
Diaporthe sp.	–	+	–	–	–	–
Eurotium rubrum	–	+	–	–	–	–
Fuckelia ribis	–	+	–	–	–	–
Fusarium incarnatum	+	+	–	–	–	–
Fusarium lateritium	–	–	+	+	–	+
Gloeosporium sp.	–	–	–	–	+	–
Gonatobotryum apiculatum	–	–	–	–	–	+
Hansfordia pulvinata	–	–	+	–	–	+
Lasmeniella sp.	–	+	–	–	–	–
Penicillium sp.	+	–	+	–	–	–
Pestalotiopsis microspora	–	–	–	+	–	+
Phomopsis arnoldiae	–	+	–	–	–	–
Phomopsis stipata	–	+	–	–	–	–
Rabenhorstia sp.	–	–	+	–	–	–
Rhinocladiella aquaspersa	–	+	–	–	–	–
Trichothecium roseum	–	–	–	–	–	+
Vermiculariopsiella indica	–	–	–	–	+	–
Vermiculariopsiella parva	–	+	–	–	+	–
Verticillium theobromae	–	–	–	–	–	+
Zygosporium minus	–	–	–	–	–	+
Unidentified Basidiomycete 1	–	–	–	–	+	–
Unidentified Basidiomycete 2	–	+	–	–	–	–
Unidentified Coelomycete 1	–	–	–	–	–	+
NSM 01	–	+	–	–	+	–
NSM 02	–	–	–	–	+	+
NSM 03	–	–	–	–	+	–
NSM 04	–	+	–	–	–	–
NSM 09	–	–	+	–	–	–
NSM 10	–	+	–	–	–	–
NSM 11	–	–	–	–	+	–
NSM 12	–	–	–	–	+	–
NSM 13	–	–	–	–	+	–
NSM 14	–	+	–	–	–	–
NSM 15	–	–	–	–	–	+
Total	3	18	8	5	14	12

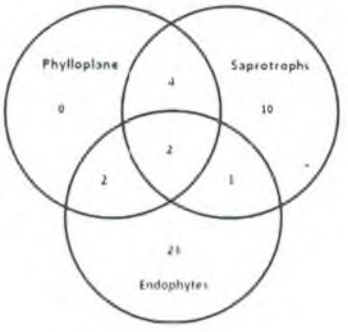

Fig. 1. Venn Diagram indicating species overlap between microhabitats

growth rate and exudates, were grouped into 15 non-sporulating morphotypes (NSM).

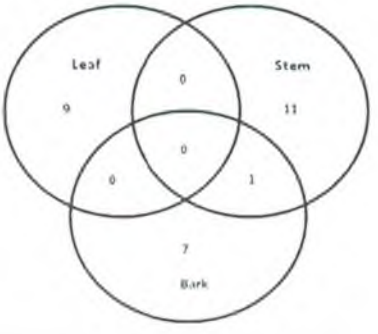

Fig. 2. Venn Diagram indicating endophytic species overlap between tissue types

The diversity was found to be less when compared to similar studies carried out on some forest plants in this region (D'Souza, 2002). Similar observations on less diversity and host specificity were made by several authors who mostly worked on documentation of endophytic mycoflora of medicinal plants (Raviraja, 2005; Bettucci & Saravay, 1993). The low diversity of associative fungi may be due to the impending chemical composition of medicinal plants.

Genera such as, *Acremonium, Aspergillus, Cladosporium, Eurotium, Fusarium, Penicillium, Pestalotiopsis* and *Verticillium* reported in the present study were also recorded elsewhere as common inhabitants of medicinal plants (Horie *et al.*, 1979; Mahesh *et al.*, 2005; Tejesvi *et al.*, 2006; Verma *et al.*, 2007). Hyphomycetous fungi were the dominant group in the study followed by non-sporulating morphotypes and the Coelomycetes. Ascomycetous diversity was found to be the least. The observation that Hyphomycetes dominated in such a study was also noted by Santos & Rodrigues (2003), Suryanarayanan *et al.*, (1998) and Mahesh *et al.*, (2005).

Phylloplane fungi reported during the study included species in the genera such as *Aspergillus, Cladosporium* and *Penicillium*. These observations are in agreement with other studies carried out on diversity of phylloplane flora (Pandey, 1990; Vardavakis, 1988; Lee & Hyde, 2002; Pereira *et al.*, 2002). As reported by several authors (Medeiros, 1988; Rodrigues, 1991; Rodrigues & Samuels, 1992; Azevedo *et al.*, 2000; Gamboa *et al.*, 2002; Johnson *et al.*, 1992; Larran *et al.*, 2001; Pereira *et al.*, 1993) species belonging to *Colletotrichum, Fusarium* and *Phomopsis* were found to be the most common endophytes in the present study. The saprophytic fungal diversity associated with the plant was represented by some of the well known tropical genera such as *Aspergillus, Cladosporium* and *Hansfordia* (Ellis, 1971; 1976; Matsushima, 1971, 1975).

In an effort to understand the interactions between the plant and fungi, different ecological niches or microhabitats such as leaf surface, internal plant tissue and senescent plant parts, in different localities and seasons were analysed. Data sets of the two sites, in a single season when taken together, overall richness of fungi in the ecological niches is evident. Among the 42 distinct isolates, 8 were phylloplane, 28 endophytes and 17 saprotrophs (Table 2) indicating that endophytes are the most abundant in species diversity followed by saprophytic and phylloplane fungi. Overlap and uniqueness between the microhabitats has been indicated using the Venn diagram (Fig.1) which shows that two species, viz. *Aspergillus fumigatus* and *Cladosporium cladosporioides*, were common in all three substrate types. Overlap between phylloplane-endophyte, endophyte-saprotroph and saprotroph- phylloplane was of 2, 1 and 4 species, respectively (Fig.1). This observation of proportionate number of overlapping species between microhabitats have been done by other workers (Davenport, 1976; Ruinen, 1961; Carroll & Petrini, 1983; Griffith & Boddy, 1988; Kowalski & Kehr, 1997; Sadaka & Ponge, 2003) as well who opined that number of fungi occurring as endophytes occur as phylloplane or latent pathogens and later as saprophytic litter fungi.

Further within endophytes, when different tissue types were compared for species overlap it was observed that, of the 28 distinct isolates, 9 were leaf endophytes, 11 stem endophytes and 7 bark endophytes. The variable number of endophytic fungal species existing in various tissue types indicates that no single tissue harbors richer endophytic diversity than the other. This result is unlike that of Radu & Kqueen (2002) who reported leaves to have higher diversity than other tissues. The overlap between leaf-bark and leaf-stem was nil while for stem-bark the overlap was of only 1 isolate. No isolates were common to all three tissue types. With low overlap in fungal

Table 3: Endophytes from *Aegle marmelos* and their percent isolation frequency

List of fungi	Dry season/ Site I				Dry season/ Site II				Wet season/ Site 1			
	Leaf	Stem	Bark	% colonization	Leaf	Stem	Bark	% colonization	Leaf	Stem	Bark	% colonization
Acremonium curvulum	–	–	–	**0**	–	–	12	**4**	–	–	18	**6**
Amerosporium sabalinum	–	–	–	**0**	–	–	–	**0**	–	16	–	**5.33**
Aspergillus fumigatus	–	–	1	**0.33**	–	–	**9**	**3**	–	–	–	**0**
Aspergillus niger	–	–	4	**1.33**	–	–	18	**6**	–	–	–	**0**
Cladosporium cladosporioides	–	–	–	**0**	24	–	–	**8**	–	–	–	**0**
Coleophoma empetri	14	–	–	**4.67**	–	–	–	**0**	–	–	–	**0**
Colletotrichum gloeosporioides	–	5	–	**1.67**	–	–	–	**0**	–	16	–	**5.33**
Diaporthe sp.	–	65	–	**21.67**	–	–	–	**0**	–	–	–	**0**
Discosporium sp.	–	–	–	**0**	–	–	–	**0**	–	18	–	**6**
Eurotium rubrum	–	1	–	**0.33**	–	–	–	**0**	–	19	–	**6.33**
Fuckelia ribis	–	1	–	**0.33**	–	–	–	**0**	–	12	–	**4**
Fusarium incarnatum	22	–	–	**7.33**	–	–	–	**0**	–	–	–	**0**
Gloeosporium sp.	–	–	–	**0**	29	–	–	**9.67**	–	–	–	**0**
Lasmeniella sp.	1	–	–	**0.33**	–	–	–	**0**	–	–	–	**0**
Ophioceras commune	–	–	–	**0**	–	–	–	**0**	33	–	–	**11**
Phomopsis arnoldiae	–	1	–	**0.33**	–	–	–	**0**	–	–	–	**0**
Phomopsis stipata	–	5	–	**1.67**	–	–	–	**0**	–	10	–	**3.33**
Phomopsis sp.	–	–	–	**0**	–	–	–	**0**	27	–	–	**9**
Rhinocladiella aquaspersa	–	4	–	**1.33**	–	–	–	**0**	–	–	–	**0**
Vermiculariopsiella indica	–	–	–	**0**	–	2	–	**0.67**	–	–	–	**0**
Vermiculariopsiella parva	–	–	–	**0.33**	16	–	–	**5.33**	–	–	–	**0**
Unidentified Basidiomycete 1	–	–	–	**0**	–	–	32	**10.67**	–	–	–	**0**
Unidentified Basidiomycete 2	–	–	42	**14**	–	–	–	**0**	–	–	–	**0**
Unidentified Coelomycete 1	–	–	–	**0**	–	–	–	**0**	19	–	–	**6.33**

List of fungi	Dry season/ Site I				Dry season/ Site II				Wet season/ Site 1			
	Leaf	Stem	Bark	% colonization	Leaf	Stem	Bark	% colonization	Leaf	Stem	Bark	% colonization
Unidentified Coelomycete 2	–	–	–	**0**	–	–	–	**0**	–	–	29	**9.66**
NSM 01	–	1	–	**0.33**	–	1	–	**0.33**	–	–	–	**0**
NSM 02	–	–	–	**0**	–	57	12	**23**	–	–	–	**0**
NSM 03	–	–	–	**0**	4	–	–	**1.33**	–	–	–	**0**
NSM 04	1	–	–	**0.33**	–	–	–	**0**	–	–	–	**0**
NSM 05	–	–	–	**0**	–	–	–	**0**	8	–	–	**2.66**
NSM 06	–	–	–	**0**	–	–	–	**0**	–	5	–	**1.66**
NSM 07	–	–	–	**0**	–	–	–	**0**	–	–	33	**11**
NSM 08	–	–	–	**0**	–	–	–	**0**	–	–	10	**3.33**
NSM 10	–	–	31	**10.33**	–	–	–	**0**	–	–	–	**0**
NSM 11	–	–	–	**0**	–	8	–	**2.67**	–	–	–	**0**
NSM 12	–	–	–	**0**	–	19	–	**6.33**	–	–	–	**0**
NSM 13	–	–	–	**0**	–	–	1	**0.33**	–	–	–	**0**
NSM 14	20	–	–	**6.67**	–	–	–	**0**	–	–	–	**0**
Percent tissue colonization	59	83	78	–	73	87	84	–	87	96	90	–
Shannon diversity Index	1.283	0.889	0.908	–	1.224	0.967	1.546	–	1.283	1.881	1.299	–
Simpson's diversity index (1-D)	0.689	0.376	0.549	–	0.683	0.514	0.756	–	0.704	0.841	0.709	–

diversity between different tissues and, as observed by Rajagopal & Suryanarayanan (2000) and Bayman *et al.*, (1997), it is said that most of the species occurring as endophytes exhibit strict tissue preference.

Endophytic fungi isolated from *Aegle marmelos* were compared for the variation in their colonization frequencies as an effect of tissue type, season and collection sites (Table 3). *Diaporthe* sp., with 21.67% colonization frequency, was the most frequently isolated endophyte during the dry season while *Ophioceras commune* and a non-sporulating isolate, with 11% colonization frequency each, were more frequently isolated during the wet season. Fungi such as *Lasmeniella* sp., *Phomopsis arnoldiae* and two other non-sporulating morphotypes appeared as singletons during the dry period while no singletons appeared in the wet season. Certain other species such as *Aspergillus fumigatus, Eurotium rubrum* and *Fuckelia ribis* occurred as singletons in the dry season while their colonization increased during the wet season. *Vermiculariopsiella parva* occurred as a singleton in Site I but at Site II its isolation frequency was higher. Arnold *et al.*, (2000) suggested that many fungi exhibited limited growth in and colonization of the internal plant tissue.

It was observed that stem tissue was most colonized in both seasons at both the collection sites (Table 3). Leaf tissue was the least colonized among the three tissues tested. Certain fungi were found to colonize similar tissues, irrespective of the site. For example, *Aspergillus fumigatus* and *A. niger* colonized the bark, *Vermiculariopsiella parva* colonized the leaf

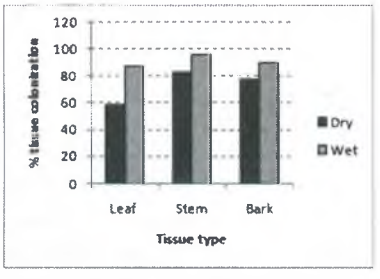

Fig. 3. Seasonal variation in percent tissue colonization

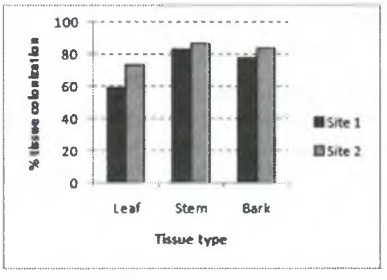

Fig. 4. Comparison in percent tissue colonization between study sites

while a non-sporulating morphotype colonized the stem tissue from both the localities. Similarly, certain others such as, *Colletotrichum gloeosporioides, Eurotium rubrum, Fuckelia ribis* and *Phomopsis stipata* colonized similar tissues irrespective of the season. Overall tissue colonization was more in the wet season as compared to the dry (Fig 3). The Site II had higher tissue colonization percentage as compared to Site I (Fig 4). Rajagopal & Suryanarayanan (2000) observed that fungal colonization in *Azadirachta indica* varied with tissue type, site and seasonality.

Several of the species occurred only in one season while certain others such as *Colletotrichum gloeosporioides, Eurotium rubrum, Fuckelia ribis* and *Phomopsis stipata* appeared throughout the year. Fungi recovered from the two sites revealed that many species occurred only at a single site while certain species such as *Aspergillus fumigatus, A. niger, V. parva* and a non-sporulating isolate occurred at both the sites studied. Verma *et al.*, (2007) had documented a distinct diversity of endophytic fungi in *Aegle marmelos*.

To understand the effect of tissue types, season and collection site on diversity of associated microfungi Shannon's and Simpson's diversity indices were calculated (Table 3). Diversity indices for each of tissue type was variable with no single tissue at all times showing higher diversity than the other. When the diversity indices were compared for the two collection sites, Site II showed higher stem and bark diversity as compared to Site I. Diversity indices of leaf was similar for the two sites. The diversity was high in wet season for all the tissue types tested as compared to the dry season. Similar to what Gangadevi & Muthumary (2006), Suryanarayanan & Johnson (2005) and Suryanarayanan *et al.* (1998) said, it was inferred that precipitation encouraged increase in endophytic diversity.

The average colonization of *Aegle marmelos* under different conditions was 82%. This results were comparable to studies carried elsewhere in the tropics where the colonization percentage varied from 30% (Rodrigues, 1994) to 90-95% (Lodge *et al.*, 1996; Gamboa and Bayman, 2001).

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