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Occurrence of non-obligate microfungi inside lichen thalli

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Five corticolous lichen species (four foliose and one fruticose) and the leaf and bark tissues of their host trees were screened for the presence of asymptomatic, culturable microfungi. Four isolation procedures were evaluated to identify the most suitable one for isolating the internal mycobiota of lichens. A total of 242 isolates of 21 fungal genera were recovered from 500 thallus segments of the lichens. Different fungi dominated the fungal assemblages of the lichen thalli and the host tissues. An ordination analysis showed that there was little overlap between the fungi of the lichens and those of the host tissues even though, considering their close proximity, they must have been exposed to the same fungal inoculum. This is the first study that compares the microfungal assemblage associated with lichens with those occurring in their substrates. It indicates that lichen thalli, apart from their obligate mycobiont, could serve as an ecological niche for certain microfungi.

Keywords: Endolichenic fungi, endophytes, foliose lichens, fruticose lichens

Lichen thalli harbour, apart from their obligate symbiotic mycobionts, other fungi that are parasitic, commensals or saprobial (Hawksworth, 1982; 1983; Honegger, 1996). Attempts to culture the mycobiont of lichens are fraught with difficulties because of the presence of such fungi (Crittenden & al., 1995; Petrini & al., 1990). Although there are several studies on the primary mycobionts of lichens (Richardson, 1999), few studies have been made regarding culturable asymptomatic microfungi existing within the lichen thalli (Girlanda & al., 1997; Peršoh, 2002; Miadlikowska & al., 2004). To understand the role of lichens as niches for such fungi, we compared the fungal assemblages of five lichen species with the phellophytes (fungi occurring in the bark tissues) and foliar endophytes of their tree hosts. We also evaluated four procedures for isolating culturable asymptomatic microfungi (called endolichenic fungi by Miadlikowska & al., 2004) associated with the lichens.

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Materials and Methods

Collections

The five lichen species (Tab. 1) were collected from the barks of trees growing in Guindy National Park, Chennai, India (13° 14′ N lat. and 80° 16′ E lon.). The erect portions of the fruticose lichen and the upper layers of the foliose lichen thalli that were not adpressed to the bark were cut with a sterile knife for investigation. In addition, the bark and leaves of the tree hosts on which the lichens grew were also sampled (Tab. 1). They were brought to the lab in sterile paper bags and processed within 5 hours of collection. The voucher specimens of the lichens were deposited at the Lichen and Bioprospecting Laboratory, M S Swaminathan Research Foundation (MSSRF), Chennai (Tab. 1).

Table 1. – Abbreviations for the lichens (with their voucher numbers), and their host trees (abbreviations for leaf and bark are given separately) used in this screening of culturable microfungi.

Lichen		Host tree	Leaf	Bark
Dirinaria picta (Sw.) Clem. & Shear (Foliose) 800/03	DP	*Vitex negundo* L.	DPL	DPB
Heterodermia diademata (Taylor) D.D. Awasthi (Foliose) 752/03	HD	*Randia dumetorum* (Retz.) Poiret.	HDL	HDB
Physcia aipolia (Ehrh. ex Humb.) Fürnr. (Foliose) 820/03	PA	*Anacardium occidentale* L.	PAL	PAB
Pyxine cocoes (Sw.) Nyl. var. *cocoes* (Foliose) 926/03	PC	*Mangifera indica* L.	PCL	PCB
Roccella montagnei Bél. (Fruticose) 928/03	RM	*Borassus flabellifer* L.	RML	RMB

Isolation of microfungi

Fresh, healthy looking lichen thalli were washed thoroughly in running water and cut into segments of 0.5 cm^2 (0.7 × 0.7 cm). For each lichen species, 100 segments were randomly selected for further investigation (endolichenic fungi). Initially, the thallus segments of *Roccella montagnei* were subjected to four surface sterilization procedures (SSP1-4) in order to ascertain the most suitable method for isolating fungi from the lichens: SSP1) the segments were merely washed in sterile water; SSP2) 20 serial washes in sterile water (90 sec per wash) under agitation (Harley and Waid, 1995); SSP3) 20 serial washes in sterile water (90 sec per wash) under agitation followed by immersing the segments in 30 % H_2O_2 (90 sec) and

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finally washed four times (15 min each) in sterile water (Girlanda & al., 1997); SSP4) the segments were dipped in 70 % ethanol for 5 sec, followed by 4 % NaOCl for 90 sec and sterile water for 10 sec (Suryanarayanan & al., 1998). The leaf segments (0.5 cm^2), cut from the midrib portion of healthy leaves were also subjected to this last mentioned treatment. The bark segments (0.5 cm^2) were washed and surface sterilized by rinsing in 75 % ethanol for 60 sec, 4 % NaOCl for 180 sec and 75 % ethanol for 30 sec (Fisher & al., 1993).

Incubation

One hundred segments of each sample (lichen thallus, leaf or bark) were plated on Potato Dextrose Agar (PDA) medium amended with an antibiotic (Chloramphenicol 150 mgl^{-1}). Ten segments of each sample were placed on 20 ml PDA medium contained in a Petri dish. The Petri dishes were sealed using Parafilm™ and incubated in a light chamber for four weeks (Bills & Polishook, 1992; Suryanarayanan, 1992). The light regimen was 12 hours of light followed by 12 hours of darkness. The incubation temperature was 26 °C. The Petri-dishes were observed periodically and the fungi which grew out from the tissues were transferred to fresh PDA slants. To prevent the rapidly growing fungi from inhibiting the slow growing strains, the former were removed following isolation and identification before they made contact with other isolates (Bills, 1996). Fungi that sporulated were identified and those that could not be identified at the species level were given code numbers in order to distinguish them from each other.

Statistical Analysis

The species diversity (H′) of the fungal assemblage was calculated following the method of Ludwig & Reynolds (1988). The similarity coefficient between any two fungal assemblages was calculated following the method of Carroll & Carroll (1978). Correspondence analysis was performed using the software BioDiversityPro (available at biodiversity@nhm.ac.uk).

Results

Using the first three isolation procedures (SSP1–4), only *Aspergillus* and *Penicillium* species could be recovered from the thallus segments of *R. montagnei* (Tab. 2). However, the fourth isolation procedure (SSP4), which was employed for isolating endophytic fungi from angiosperm leaves (Suryanarayanan & al., 1998), resulted in the isolation of a different fungal spectrum from the lichen thallus

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(Tab. 2) indicating that this method eliminated surface borne fungi (such as *Aspergillus* and *Penicillium*) and facilitated the growth of the cryptic, culturable fungi borne within the lichen thallus. Hence, we used this surface sterilization procedure for the rest of the lichen species as well.

Table 2. – Number of isolates of endolichenic fungi (in alphabetical order) isolated from the lichen *Roccella montagnei* using the four different surface sterilization procedures (SSP1-4).

Endophyte	**SSP 1**	**SSP 2**	**SSP 3**	**SSP 4**
Aspergillus niger van Tiegh.	23	9		
Aspergillus sp. 1		2		
Aspergillus sp. 2		1		
Botrytis sp.				3
Chaetomium sp.				1
Cladosporium sp.				3
Lasiodiplodia theobromae (Pat.) Griffon & Maubl.			1	
Paecilomyces sp.				3
Penicillium sp. 1		1	1	
Phialophora sp.				2
Rhizopus sp.				5
Sporormiella intermedia (Auersw.) Ahmed & Cain				11
Sterile form LN1				1
Trichoderma sp.	2			
Xylariaceous form 1			3	3
Xylariaceous form 2				2

A total of 242 isolates of fungi belonging to 24 species of 21 genera were recovered from 500 thallus segments of the lichens. In most of the cases, the diversity of endolichenic fungi was higher than that of the endophytes or phellophytes of the tree host (Tab. 4). *Sporormiella intermedia* dominated the endolichenic assemblage of three lichen species. Usually, the fungal species dominating the assemblage of a lichen was absent or present in low frequency in the bark or leaf tissue of its host (Tab. 3 and 4). *Vice versa*, fungi such as *Phomopsis* sp. and *Phyllosticta capitalensis* dominating the endophyte or phellophyte assemblage of a host tissue, were either absent or present in low frequency in the lichen thalli growing on the respective tree (Tab. 3 and 4). An ordination analysis showed that the endolichen assemblages were distinct from those of the bark and the leaf fungal assemblages (Fig. 1). Furthermore, the similarity index showed that the overlap between the endolichenic fungi and the phellophytes or endophytes ranged from 2–28 % only (Tab. 5).

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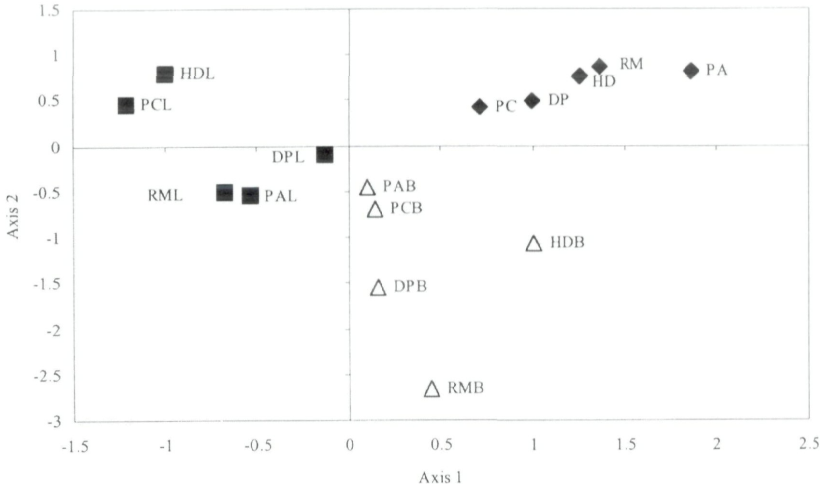

Fig. 1. Correspondence analysis of the culturable microfungi, endophytes and phellophytes isolated from five lichen thalli and the leaves and barks of their hosts, respectively. Refer to Tab. 1 for codes. Only fungi that had a colonization frequency >5 % were used in this analysis. Black diamonds: lichen samples; triangles: bark samples, black squares: leaf samples.

Discussion

A lichen, the bark on which it grows and the leaf of its tree host are in close proximity and are consequently, exposed to virtually the same inoculum. However, our results indicated that the overall similarity between the fungal assemblages of the three entities was low. It is likely that factors such as the suitability of the substrate and the competitive potential of the fungi on the respective substrates play a role in the development of the fungal communities in different tissues. Such a distribution is likely to reduce interspecific competition among fungi (Kumaresan & Suryanarayanan, 2001). It is pertinent that Petrini & al. (1992) suggest that tissues of plant hosts function as microhabitats as far as endophyte infections are concerned. Studying the endophyte distribution in the leaf, bark and propagule tissues of the mangrove tree *Rhizophora apiculata* Bl., Kumaresan & al. (2002) found that different fungi dominated the assemblages of these tissues. In the present study, it was also observed that the dominant fungus was different for the lichen and its host tissues (Tab. 4). Thus, it appears that the endolichenic fungi are not mere contaminants or casual residents of lichen thalli. Although the endolichenic fungi isolated in this study are not unique and have been recovered as endophytes from different plants (for eg. Suryanarayanan & al., 2002), our results confirm that lichens har-

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bour microfungi apart from their obligate mycobionts (Miadlikowska & al., 2004; Girlanda & al., 1997). Miadlikowska & al. (2004) compared the endolichenic fungal assemblages of *Peltigera* from forests in North Carolina and Costa Rica and, based on ITS rDNA sequence similarity, concluded that endolichenic fungi are distinct from the lichenicolous fungi and that they are phylogenetically related to the endophytic fungi harboured by plants. These results and the staggering number of fungi obligately occurring on lichens (the lichenicolous fungi) (Lawrey & Diederich, 2003; Hawksworth, 2001) together substantiate the view of Suryanarayanan & Hawksworth (2005) that lichens be routinely included in fungal diversity estimation studies. Detailed histological and molecular studies on lichens of different habitats are needed to know if these fungi actually grow inside the lichen thalli or remain in a dormant state.

The presence of endolichenic fungi in the lichen thalli has been confirmed by plating surface sterilized thallus tissues on growth medium (Girlanda & al., 1997). Some of the metabolites of lichens are antibiotic in nature (Lawrey, 1987; Rundel, 1978) and the sensitivity of fungi to these metabolites differ (Torzilli & Lawrey, 1995). Obviously, the fungi occurring inside the lichen thalli are, in their respective states, unaffected by these metabolites. There are no studies regarding the effects of lichen metabolites on the endolichenic fungi. Our preliminary observations showed that the foliar endophytes of *Borassus flabellifer* are inhibited when grown on a medium containing an aqueous extract (2 %) of *Rocella montagnei*. This perhaps explains the differential distribution of microfungi among the lichen and its tree host tissues. Further experiments with fungal strains isolated from lichens and their hosts in the presence of lichen metabolites are needed to confirm this observation.

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Table 3. – Number of isolates of endolichenic fungi, endophytes and phellophytes isolated from five lichen thalli and the leaves and barks of their hosts respectively. Refer to Tab. 1 for abbreviations. Only fungi with a colonization frequency of >5% are mentioned in the table. Fungi with a colonization frequency of <5% (the host tissue codes are given as superscripts) were: *Sordaria* sp.$^{\text{PA, HD}}$, *Sporormiella minima* (Auersw.) Ahmed & Cain$^{\text{PA, HDL}}$, Xylariaceous form 2$^{\text{RM}}$, *Colletotrichum* sp. 2$^{\text{PCL}}$, *Phoma* sp.$^{\text{DPL, PAL, RML}}$, *Alternaria* sp.$^{\text{RML}}$, *Arthrinium* sp.$^{\text{DP, HD, PA, PCB}}$, *Curvularia* sp.$^{\text{RML}}$, *Drechslera halodes* Subram. & Jain$^{\text{PCL}}$, *Graphium* sp.$^{\text{HD, HDL}}$, *Nodulisporium* sp.$^{\text{PA, PCL}}$, *Oidiodendron* sp.$^{\text{HD}}$, *Penicillium* sp. 1$^{\text{PC, DPL, PAL, PCL, HDB}}$, *Penicillium* sp. 2$^{\text{HD}}$, *Phialophora* sp.$^{\text{HD, PA, PC, RM, RML, PAB, PCB}}$, *Torulomyces* sp.$^{\text{DP, DPL, RMB}}$, Sterile form LN1$^{\text{PA, RM, RML, PCB}}$.

	DP	HD	PA	PC	RM	DPL
Chaetomium sp.	4	3	6	1	1	
Glomerella cingulata Spauld. & H. Schrenk.			1			
Sporormiella intermedia (Auersw.) Ahmed & Cain	5	16	12		11	
Xylariaceous form 1	3	13	2	14	3	
Colletotrichum gloeosporioides (Penz.) Sacc.						
Lasiodiplodia theobromae (Pat.) Griff. & Maubl.	2		3	1		
Pestalotiopsis sp.	5					
Phomopsis sp.			2			10
Phyllosticta capitalensis P. Henn.					4	
Aspergillus niger van Tiegh.						
Botrytis sp.	1		2	4	3	
Cladosporium sp.	4	6	2	1	3	4
Fusarium sp.						
Humicola sp.		2	32	1		
Nigrospora oryzae (Berk. & Br.) Petch.	3	3	10			2
Paecilomyces sp.	2	9		5	3	3
Trichoderma sp.						
Rhizopus sp.					5	

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HDL	PAL	PCL	RML	DPB	HDB	PAB	PCB	RMB
							2	
10		1						
19	1			5	5	7	3	
29	3	8	3		2		4	
	1		2	7	12	8	3	10
10						3		
2	21	18	51	3	3	19	4	6
82	7	54	9	2				
	1			1				19
						5	1	
	4					1	3	2
	5		1	2	3		1	1
						2		
				1	1			4
6								2
		1		7			4	11
			2					

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Table 4. – Total number of isolates and species and their diversity values for microfungi recovered from five lichen thalli and their host tissues (leaf and bark are given separately). Refer to Tab. 1 for abbreviations.

Abb.	**No. of Isolates**	**No. of Species**	**Diversity index (H')**	**Dominant fungus**
Lichen				
DP	36	11	2.32	*Sporormiella intermedia*, *Pestalotiopsis* sp.
HD	61	12	2.10	*Sporormiella intermedia*
PA	80	16	2.06	*Humicola* sp.
PC	31	10	1.76	Xylariaceous form 1
RM	34	10	2.04	*Sporormiella intermedia*
Leaf				
DPL	26	8	1.77	*Phomopsis* sp.
HDL	164	9	1.56	*Phyllosticta capitalensis*
PAL	45	10	1.71	*Phomopsis* sp.
PCL	89	8	1.22	*Phyllosticta capitalensis*
RML	77	11	1.33	*Phomopsis* sp.
Bark				
DPB	28	8	1.86	*Lasiodiplodia theobromae*, *Trichoderma* sp.,
HDB	29	8	1.74	*Lasiodiplodia theobromae*
PAB	47	8	1.72	*Phomopsis* sp.
PCB	29	12	2.36	*Colletotrichum gloeosporioides*, *Phomopsis* sp., *Trichoderma* sp.
RMB	56	9	1.80	*Aspergillus niger*

Table 5. – Similarity coefficient (Carroll & Carroll, 1978) for endolichenic fungi and the phellophytes and endophytes of the respective tree hosts (Refer to Tab. 1 for abbreviations).

	Similarity values (%)*	
Lichen	**Host Bark**	**Host Leaf**
Dirinaria picta	20	28
Heterodermia diademata	14	20
Physcia aipolia	22	9
Pyxine cocoes	27	2
Roccella montagnei	9	7

* Value can range from 0 % for complete dissimilarity to 100 % for complete similarity.

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