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DIVERSITY OF PHYLLOPLANE MYCOBIOTA OF *AVICENNIA MARINA* (FORSSK.) VIERH. AND *RHIZOPHORA MUCRONATA* POIR. AT INDUS DELTA, SINDH COAST, PAKISTAN

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ABSTRACT

Phylloplane fungal assemblages of two mangrove species *Avicennia marina* and *Rhizophora mucronata* were investigated at two sites each. Altogether twenty-three fungal species and 16 genera were recorded. Greater number of species and genera were recorded for *Rhizophora mucronata*. Among the sites studied, Keti Bunder showed the highest number of fungal species and genera associated with the phylloplane. In general, the phylloplane mycobiota was dominated by the genera *Aspergillus*, *Cladosporium*, *Alternaria* and *Fusarium*. The genus *Aspergillus* was represented by six species. *Aspergillus niger*, *A. fumigatus* and *A. flavus* were most abundant.as measured by CFUs/cm^2 of phylloplane.

Species diversity and its components for the fungal communities were estimated. It was found that the general diversity (H′) was slightly but consistently higher for *Rhizophora mucronata* compared to *Avicennia marina*. However, equitability (J′) was more or less equal for the two mangrove species. Regarding sites, equitability was slightly higher for Rehree Island. High qualitative similarities of phylloplane mycobiota were found between species and between sites. The correspondence between air-spora and the phylloplane fungal assemblages is discussed.

Key-words: Phylloplane fungi, mangrove species, *Avicennia marina*, *Rhizophora mucronata*, Indus delta, Pakistan.

INTRODUCTION

Phylloplane, the surface of plant leaves is a complex microhabitat characterized by the presence of various micro-organisms including bacteria, filamentous fungi and yeasts (Levetin and Dorsey, 2006). The leaf surface biota comprises of pathogens, saprobes and epiphytes. Lee and Hyde (2002) recognized two groups of phylloplane mycotbiota: residents and casuals (Norse, 1972). Resident fungi can reproduce and grow on surface of healthy leaves without causing noticeable changes in the host plant. Whereas, casuals land on the phylloplane but do not grow (Leben, 1965). Several workers have investigated the phylloplane microbial assemblages of different plant species (Breeze and Dix, 1981; Mishra and Dickinson, 1981; De Jager *et al*., 2001; Andrew *et al*., 2002; Bakker *et al*., 2002; Ososno, 2002; Osono *et al*., 2004; Kishore *et al*., 2005; Levetin and Dorsey, 2006). It has been noted (Nicholson, 1972) that the microorganisms occurring on the leaf surfaces are also common in either soil or air. Thus it may be debated that these microbiota are merely casual contaminants (constituted by random mingling of species) and do not constitute an organized community (Sugihara, 1980; Anderson and Calmay, 2004; Asensio *et al*., 2007; Ferreira and Petrere, 2008; Meyer and Leveau, 2012). Nicholson (1972) conducted a series of experiments to test this notion and demonstrated that the populations of microorganisms interact, grow and multiply on the phylloplane and form organized communities. The interaction of microbial populations, in particular, plays an eminent role in determining the structure and composition of the phylloplane microbial community. The phylloplane microorganisms are of considerable importance as some of them are antagonists to pathogenic microorganisms infecting the plant species (Blakeman, 1991; El-Said, 2001; Mandhare and Suryawanshi, 2009). The interaction of microbial populations, in particular, plays an eminent role in determining the structure and composition of the phylloplane microbial community. The competitive abilities or microbial populations composing the communities on the leaf surfaces can be modified by various inhibitory agents such as heavy metal concentrations present in the leaves (Smith, 1977).

A few investigations have been conducted on the phylloplane microbial assemblages on mangrove leaves. Newell (1976) examined the succession of fungi on the leaves of the red mangrove (*Rhizophora mangle* L.). Kuthubutheen (1981) investigated the fungi associated with leaves and other aerial parts of Malaysian mangrove plants. Kuthubutheen (1984) examined the leaf surface fungi associated with two mangrove species *Avicennia marina* and *Rhizophora mucronata* in Malaysia. Sivakumar and Kathiresan (1990) studied the phylloplane fungi of mangrove species in the east coast of India. Lee and Hyde (2002) evaluated study methods of phylloplane fungi of mangrove species *Candelia candel* and *Aegiceras corniculatum* using light microscopy and scanning electron microscopy (SEM). Sridhar (2009) evaluated the fungal diversity of Pichavaram mangroves, Southeast coast of

India. Naikwade *et al*., (2012) investigated the phylloplane mycobiota associated with the mangrove plant *Ceriops tagal.* Thatoi *et al*., (2012) worked on the microbial diversity of mangrove soils of Bhitarkanika, Odisha, India. However, no work has yet been done on the phylloplane mycobiota of the mangroves of Pakistan coast.

The objectives of the current investigation were 1) to assess the abundance and composition of phylloplane fungi of two mangrove species *Avicennia marina* (Forssk.)Vierh. And *Rhizophora mucronata* Poir., 2) to evaluate any site differences in the phylloplane mycobiota of the two selected mangrove species, and 3) to estimate the species diversity and its components for the fungal assemblages under study.

MATERIALS AND METHODS

Sampling:

The sampling of *Avicennia marina* leaves was performed at Sndspit and Rehri Island while that of *Rhizophora mucronata* at Keti Bunder and Rehreei Island. The three sites are situated in southern Sindh, Pakistan. Sampling was conducted during May - July, 2011. The leaves were collected from 1.2 to 1.5 m above ground or water level, and were seldom immersed even during high tide. From each site 10 leaves of the mangrove species were collected from 5 randomly chosen plants. On an average, the size of *A. marina* leaf-blade was 3.6. × 11.5 cm and that of *R. mucronata* 6.2 × 12.1 cm. Collection of leaf was restricted to healthy and green leaves to ensure only true phylloplane fungi. Only photosynthetically active (non-senescent) leaves were sampled. Any disturbance of the experimental leaves was avoided by cutting the petiole and adjacent branches, the collected leaves were immediately brought to laboratory in sterile polythene bags. The assay of mycobiota was carried out within 24 h of sampling.

Fungal cultures and assessment of mycobiota:

For each leaf four 1 cm^2 areas were cut with a sterile stainless steel template with 1 cm^2 opening to ensure consistent leaf sample area and care was taken to avoid the central midrib of the leaf. The four leaf sections were rinsed together in 2 ml sterile distilled water by vortexing for 1 minute (Levitin and Dorsey, 2006). A 0.5 ml aliquot of the suspension was plated onto Czapex Dox Agar (CDA) medium, in 9 cm diameter sterile Petri plates, supplemented with Penicillin and streptomycin sulphate. After incubation at 28° C, the plates were examined for number of fungal colonies, and then observed under a microscope. Most isolates were obtained after a few days of incubation (generally 3 days), but plates were checked over several weeks to allow isolation of slow growing fungi. Developing fungal colonies were sub-cultured into pure isolates and identified by their microscopic morphology and colony characteristics using standard mycological literature (Thom and Rapper, 1945; Booth, 1971, Domsch *et al*., 1980 , Barnett and Hunter, 1998). Results were expressed as colony forming units (CFUs)/cm^2 of leaf area. Four replicates were kept for each species at each site. A. two-factor analysis of variance (FANOVA) was performed for the abundant fungal species separately, followed by Fisher's least significant difference (LSD) test (Zar, 2008). The program for factorial analysis of variance (FANOVA) was developed by the first author (S.S.S.) in C++.

Measurement of diversity and similarity

Diversity indices

A number of diversity indices have been proposed to measure diversity (Magurran, 2004). Diversity indices represent a useful means for quantifying community diversity and have been instrumental in revealing the microorganism diversity associated with the phylloplane communities (Thomas and Shattock, 1986; Joshi, 2008). Several diversity indices were employed to compare treatment effects. Various diversity measures estimate different aspect of community structure. The general species diversity of the fungal communities was measured by the generally accepted Shannon–Wiener information theory function:

$$H' = - \sum P_i \log P_i \qquad i=1 \ldots . S$$

Where H' is the general species diversity and P_i the proportion of total number of CFU for fungal species belonging to the i*th* species and S equals the total number of species in the assemblage (Shannon and Weaver, 1963). The variance of general diversity Var (H') was calculated in accordance with Magurran (2004), as follows:

$$\text{Var}\,(H') = \sum P_i\,(\log P_i)\,2 - (\sum \mathrm{P_i} \log P_i)\,2 / N + (S\text{-}1)/\,2N^2 \quad i=1 \ldots S$$

The general diversity incorporates two components of diversity: species richness, which expresses the number of species S as a function (ratio) of the total number of individuals N; and equitability that measures the evenness of allotment of individuals among the species (Magurran, 2004). The equitability component of diversity and its variance were measured in accordance with Pielou (1975):

$$J' = H' / H'_{\max} = H' / \log S$$

The equitability index $J'$is the ratio between observed H' and maximal diversity $H'_{max.}$: Variance of equitability was estimated as:

$$\text{Var}(J') =: (H') / (\log S)^2$$

Non-parametric estimates of species richness were obtained in two different ways: (i) The jackknife estimate was obtained in accordance with Burnham and Overton (1978) and Heltshe and Forrester (1983). This estimate relies on the number of species found ($\hat{S}_{obs}$); the number occurring in only one sample (U); and n; the number of samples collected, as follows:

$$\hat{S}_{jack} = \hat{S}_{obs} + U\,(n\text{-}1/n)$$

The bootstrap estimator derived by Smith and van Belle (1984) was calculated as follows:

1. With replacement, n samples were randomly selected and the following resample value calculated from the total available

$$\hat{S}_{boot} = \hat{S}_{obs} + \sum (1- P_i)^2$$

where P_i is the proportion of the n that has species i present.

2. Step one was repeated a large number of times (say 100) and the mean of $\hat{S}_{boot}$ computed. Both jack-knifing and bootstrapping allow estimation of improved statistics, taking cognizance of rare species that were not included in the sample.

Dominance concentration (complement of diversity) was measured by using Simpson's index (Southwood and Henderson, 2000) as:

$$D = \sum \{[\, n_i\ (n_i\text{-}1)] /\ [N(N\text{-}1)]\ \} \qquad i = 1\ldots S$$

in which n_i number of CFU for a fungus

For the computation of diversity indices and their variances and the dominance concentration, a program package was developed by one of us (S.S.S.) in C++ and is available from the senior author at a nominal cost.

Measurement of Similarity:

Similarity between fungal assemblages was computed qualitatively using Sorensen's similarity coefficient (Kenkel and Booth, 1992) as follows:

$$C_{jk} = [2a / (a + b + c + d)] \text{ X } 100$$

Where a, b, c and d are the usual notations of the contingency table. The program SIMIL for computation of similarity matrix using various similarity indices was developed by the first author in C++ and is available on request.

RESULTS AND DISCUSSION

Fungal abundances in terms of CFU/cm^2 for the two mangrove species *Rhizophora mucronata* and *Avicennia marina* at two localities each are given in Table 1. The highest numbers of fungal species (23) were recorded at Keti Bunder on the phylloplane of *Rhizophora mucronata*. Greater number of species and genera were found on the phylloplane of *R. mucronata* compared *to A. marina.* Whereas the lowest number of species (18) were recorded from *Avicennia marina* growing at Rehree Island.

In general, the phylloplane mycobiota was dominated by the genus *Aspergillus.* In particular, *A. niger* and *A. flavus* were found to be the dominant species. *Aspergillus niger* showed significant difference in abundance with respect to mangrove species ($P<0.05$) and site ($P<0.05$). *Aspergillus flavus* did not exhibit significant difference with regard to mangrove species but differed significantly with site ($P<0.05$). *A. fumigatus* showed significant difference in abundance with respect to mangrove species ($P<0.05$). Moreover, *Aspergillus* was represented by six species in the study sites and the mangrove species. Mehdi and Saifullah (1992) also reported high abundance *Aspergillus* species (i.e., *A. niger* and *A. flavus*) on the phylloplane of *Avicennia marina* growing at Clifton and Korangi Creek. *Penicillium chrysogenum* had significantly greater density on the phylloplane of *R. mucronata* compared to *A. marina* ($P<0.01$). *A. wanti*, *Memnoniella* sp., *Paecilomyces* sp. and *Phoma* sp. occurred solely on *Rhizophora mucronata.* On the other hand, all species that occurred on the phylloplane of *Avicennia marina* were also recoded for the phylloplane of *Rhizophora mucronata.* In the present study *Alternaria alternata* was recorded from both the mangrove species, by contrast Mehdi and Saifullah (1992) have reported *Alternaria maritima* from the leave of *Avicennia marina* (July to September). Naikwade *et al*., (2012) recorded 9 different species of *Aspergillus* from the phylloplane of a mangrove species *Ceriops tagal* (Pers.); they also found *Alternaria alternata* on the phylloplane. The present results corroborate the findings of earlier workers with regard to phylloplane mycobiota of mangrove species. Kuthubutheen (1981) working on 9 species of mangroves reported fungal species included in the genera like *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium* and *Trichoderma* which have also been reported in the present study. The fungal assemblages sampled comprised of many pioneer species that colonize the

phylloplanes initially and subsequently their density increases substantially (Dix and Webster, 1985). The pioneer species tend to be fast growing, short-lived, and capable of rapid and widespread dispersal (Luckzkovich and Knowles, 2000). Thus profusely sporulating fungi like *Aspergillus*, *Penicillium* and *Cladosporium* were predominant. *Fusarium* species found (*F. oxysporum*, *F. Solani* and *F. .moniliformis*) were presumably non-pathogenic and occurred simply as epiphytes as no visible pathogenecity symptoms were observed. When diversity of fungal assemblages were measured, it was found that the general diversity (H′) was slightly but consistently higher for *Rhizophora mucronata* compared to *Avicennia marina* (Table 2). Equitability component of diversity (J′) was more or less equal for the two mangrove species. With respect to site, equitability was slightly higher for Rehree Island. Variances of diversity and equitability were consistently low for both species and the sites. Species richness (d) was highest for *Rhizophora mucronata* at Keti Bunder while it was lowest for *Avicennia marina* at Sandspit. Jakknife ($\hat{S}_{jack}$) and bootstrap ($\hat{S}_{boot}$) estimates of total number of phylloplane assemblages were consistently higher than the actual number of observed fungal species as these include those rare species that were not included in the sample. Dominance concentration (D) was found to vary inversely with the general diversity (H).

Table 1. Abundances (CFUs/ml) of fungal species recorded on leaf surface of *Rhizophora mucronata* and *Avicennia marina* at localities each.

Species	***Rhizophora mucronata***		***Avicennia marina***	
	Keti Bunder	Rehree Island	Rehree Island	Sandspit
Aspergillus niger	10	14	18	13
A. flavus	5	25	4	17
A. fumigatus	15	20	9	17
A. terrius	3	2	5	4
A. candidus	2	4	1	-
A. wanti	1	-	-	-
Acromium sp.	3	5	2	4
Alternatia alternata	7	9	-	1
Fusarium moniliformis	2	-	2	1
F. oxysporum	18	13	15	9
Rhizopus stolonifer.	2	3	4	1
Cladosporium cladosporiodes	16	14	-	-
Cladosporium globosum	4	1	3	3
Curvularia sp.	2	5	16	25
Penicillium chrysogenum.	20	17	2	5
Penicillium citrinum	-	2	3	-
Glycladium sp.	16	7	-	-
Memnoniella sp.	1	-	-	2
Mucor hiemalis	1	1	3	2
Mycelia sterilia (white)	-	2	1	1
Mycelia sterilia (yellow)	2	-	-	1
Trichoderma viride	-	4	7	12
Fusarium solani	1	-	5	1
Paecilomyces sp.	1	1	-	-
Phoma sp.	1	1	1	-
S (number of species)	23	20	18	19
Number of genera	16	13	11	13

A variety of environmental factors are known to affect fungal diversity (Stanwood, 2009). These factors include temperature, humidity, rainfall, dew, wind velocity and direction. In addition, intrinsic factors of the leaf also play an important role in fungal composition and diversity. It is interesting to note that the dominant phylloplane fungal species belonging to *Aspergillus*, *Alternaria*, *Cladosporium* were also the dominant species of airborne fungi (Afzal *et al*., 2005; Rao *et al*., 2009). Based on some degree of correspondence between phylloplane and airborne mycobiota, it seems that the airborne mycobiota plays an eminent role in the formation of fungal assemblages

associated with the phylloplane. By contrast, Levetin and Dorsey (2006) asserted that the leaf-surface fungi are the major contributor to the airborne mycobiota. Those taxa with an airborne dispersal are the principal contributors in this respect. Based on the estimated concentrations of the two tree species *Ulmus* and *Quercus*, they calculated that 19% of the air-spora was contributed by the phylloplane fungi. However, the air spora is also contributed by the soil surface and the decaying or other organic waste lying on the land surface through winds and gale. Thus, further studies are needed to determine the role of phylloplane mycobiota to the air spora.

Table 2. Species diversity (*H′*), variance of diversity Var(*H′*), equitability (*J′*), variance of equitability Var (*J′*), species richness d and dominance (D) and jackknife and bootstrap estimates of total number of species.

Diversity measures	***Rhizophora mucronata***		***Avicennia marina***	
	Keti Bunder	Rehree Is.	Rehree Is.	Sandspit
Species diversity (*H′*)	2.635	2.589	2.519	2.441
Variance of diversity var(*H′*)	0.006	0.005	0.002	0.007
Equitability (*J′*)	0.840	0.864	0.871	0.829
Variance of Equitability var(*J′*)	0.0006	0.0005	0.0008	0.0007
Species richness (d)	1.979	1.690	1.791	1.720
Dominance (D)	0.086	0.090	0.095	0.106
Total # of species ($\hat{S}_{jack}$)	27.7	25.8	21.5	22.4
($\hat{S}_{boot}$)	24.3	23.6	19.5	20.5

Table 3. Similarity between fungal assemblages on phylloplanes of *Rhizophora muctronata* and *Avicennia marina* at two localities for each mangrove species.

Species/Sites	***Rhizophora mucronata***		***Avicennia marina***	
	Keti Bunder	Rehree Island	Rehree Island	Sandspit
R.mucronata Keti Bunder	X	80.95	80.95	75.0
R. mucronata Rehree Is.	_	X	72.00	72.22
A. marina Rehree Is.	_	_	X	73.68
A. marina Sandspit	_	_	_	X

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