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DIVERSITY OF PHYLLOPLANE MYCOBIOTA OF TWO MANGROVE SPECIES *CERIOPS TAGAL* AND *AEGICERAS CORNICULATUM* UNDER NATURAL AND GREENHOUSE CONDITIONS

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ABSTRACT

The investigation focuses on composition and diversity of phylloplane mycobiota of two mangrove species *Ceriops tagal* (Perr.) C.B. Rob. and *Aegiceras corniculatum* (L.) Blanco from Pakistan coast and greenhouse grown plants. Altogether twenty-nine fungal species of seventeen genera were recorded from phylloplane of the two mangrove species. Phylloplane mycobiota from greenhouse plants showed lesser number of species. Greater number of species and genera were recorded for *Ceriops tagal* compared to *Aegiceras corniculatum.* In general, the phylloplane mycobiota was dominated by the genera *Aspergillus*, *Fusarium* and *Cladosporium.* The genus *Aspergillus* was represented by seven species of which *Aspergillus niger*, *A. terreus* and *A. flavus* were most abundant measured by CFUs/cm^2 on the 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 *Ceriops tagal* compared to *Aegiceras corniculatum.* However, equitability (J') was slightly higher for *Aegiceras corniculatum*. Regarding sites, equitability was slightly higher for Sonmiani and Keti Bunder but lower for plants grown in the greenhouse. The mycobiota assemblages, in general, were dominated by the genus *Aspergillus*. High qualitative similarities of phylloplane micro fungal assemblages were demonstrated between mangrove species and between sites. Generally, the diversity was lower for the phylloplane fungal assemblages of the greenhouse.

The relationship between air-spores and the phylloplane fungal assemblages showed a great deal of correspondence.

Key-words: Fungal diversity, phylloplane microflora, mangrove species, *Aegiceras corniculatum, Ceriops tagal*

INTRODUCTION

The above-ground plant surfaces such as those of leaves are together called as phyllosphere or phylloplane and are complex microhabitats characterized by the presence of various micro-organisms including bacteria, filamentous fungi, yeasts and algae (Lindow and Brandl, 2003; Levetin and Dorsey, 2006). The leaf surface biota comprises of pathogens, saprobes and epiphytes. Two groups of phylloplane mycobiota have been recognized by Lee and Hyde (2002) known as : 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). Phylloplane is characterized as a hostile environment since the availability of water, in solution and nutrient regime varies periodically as well as seasonally (Lindow and Leveau, 2002). The phylloplane microorganisms can have a significant bearing on the host plant by acting as pathogens, natural antagonists or serving as plant growth promoters (Blakeman, 1991; Andrews, 1992; El-Said, 2001; Mandhare and Suryawanshi, 2009). The ability to harbour a great number of natural antagonists of some significant plant pathogens on leaf surface is of considerable economic benefit in the biocontrol of such plant pathogens (Dighton, 2003; Yadav *et al*., 2011; Shamsi *et al*., 2012). Numerous studies have been conducted on the phylloplane microbial assemblages of different plant species (Lindsey and Pugh, 1976; Breeze and Dix, 1981; Mishra and Dickinson, 1981; De Jager*et al*., 2001; Andrews *et al*., 2002; Bakker *et al*., 2002; Ososno, 2002; Osono *et al*., 2004; Kishore *et al*., 2005; De Costa *et al*., 2006; Levetin and Dorsey, 2006). Nicholson (1972) noticed that the microorganisms prevailing on the leaf surfaces also commonly occur either in the soil or air. Thus it was doubtful whether 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; Ferreira and Petrere, 2008; Meyer and Leveau, 2012).With the help of suitable experiments, Nicholson (1972) demonstrated that the populations of microorganisms do interact, grow and multiply on the phylloplane and are organized into communities. The interaction of microbial populations, in particular, plays an eminent role in determining the structure and composition of the phylloplane microbial community. The density and diversity of the microbial populations change with time depending on host plant

species, its growth stage, growing season and changes in physico-chemical characteristics of the leaf surfaces (Hirano and Upper, 2000; Mercier and Lindow, 2000). The interaction of microbial populations, particularly competition, which is often strong, plays a prominent role in determining the structure and composition of the phylloplane microbial community. The competitive abilities of microbial populations comprising the communities on the leaf surfaces can be altered by various inhibitory agents such as heavy metal concentrations present in the leaves or as a result of chronic exposure to gases like SO_2 and O_3 (Smith, 1977; Fenn*et al*., 1989).

Mangroves are important ecosystems as they are highly productive, they protect the coast and harbour both marine vertebrates and invertebrates. Some studies have been conducted on the phylloplane microbial assemblages on mangrove plants of Indo-Pakistan coast. Succession of phylloplane mycobiota was examined by Newell (1976) on the leaves of the red mangrove (*Rhizophora mangle* L.). Kuthubutheen (1981) studied the fungi associated with the phyllosphere of Malaysian mangrove species. Kuthubutheen (1984) reported the phylloplane fungi of two mangrove species *Avicennia marina* and *Rhizophora mucronata* in Malaysia. Sivakumar and Kathiresan (1990) investigated the phylloplane fungi of mangrove species in the east coast of India. Lee and Hyde (2002) compared the study methods of phylloplane fungi of mangrove species *Candelia candel* and *Aegiceras corniculatum* using light and scanning electron microscopy (SEM). Tariq *et al*., (2006) reported the occurrence of fungi on the leaves of three mangrove species from a part of Pakistan coast. Sridhar (2009) assessed the fungal diversity of Pichavaram mangroves, Southeast coast of India. Naikwade *et al*., (2012) reported the phylloplane mycobiota associated with the mangrove species *Ceriops tagal.* Thatoi *et al*., (2012) assessed the microbial diversity of mangrove soils of Bhitarkanika, Odisha, India. Shaukat *et al*., (2013) determined the composition and diversity of phylloplane mycobiota of two mangrove species, namely *Avicennia marina* and *Rhizophora mucronata* from Indus delta, Sindh. Because mangrove detrital system is an important fundamental source of nutrient release in the food webs of mangrove ecosystems the study of the microorganisms associated with leaves and litter that eventually undergo decay are of great significance from the standpoint of nutrient cycling.

The study was undertaken with the following objectives: 1) to assess the abundance and composition of phylloplane fungi of two mangrove species *Ceriops tagal* (Perr.) C.B. Rob. and *Aegiceras corniculatum* (L.) Blanco from the coastal areas of Pakistan and also from a greenhouse located at Karachi University Campus, 2) to quantify the similarity of phylloplane microfungal composition of the assemblages of the two selected mangrove species under natural and greenhouse conditions, and 3) to measure the species diversity and its components (species richness and equitability) for the fungal assemblages under investigation.

MATERIALS AND METHODS

Sampling:

Sampling was performed during June – July, 2012 at three different localities for each of the mangrove species. The sampling of *Ceriops tagal* (Perr.) C.B. Rob. leaves was conducted at Sonmiani, Keti Bunder and the greenhouse (Karachi University Campus) while that of *Aegiceras corniculatum* (L.) Blanco was conducted near Korangi Creek, Keti Bunder and the green house. The leaves were collected from 0.8.0 to 1.5 m above ground and in natural conditions they were seldom immersed even during high tide. From each site 5leaves of each mangrove species were collected from 3 randomly chosen plants. *Ceriops tagal* and *Aegiceras carniculatum* plants obtained from Sindh Forest Department were grown in the greenhouse of the Institute of Environmental studies. The greenhouse is constructed by green nylon netting and a number of plant species besides the mangrove species are kept and raised in it. At the time of sampling the mangrove plants were 1 to 1.6- year old. The same sampling procedure was followed for greenhouse mangrove plants as that used for plants at naturally growing mangrove sites. 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 24h 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 glass Petri plate, 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 5-6 days), but plates were checked over several weeks to allow isolation of slow growing fungi. Each colony was assumed to have originated from a unit propagule. 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; Ellis and Ellis, 2009). Results were expressed as colony forming units (CFUs)/cm^2 of leaf area. Five replicates were kept for each species at each site. A two-factor analysis of variance (ANOVA) was performed for the abundant fungal species separately, followed by Fisher's least significant difference (LSD) test and Duncan's multiple range test (Zar, 2009). The program for factorial analysis of variance (FANOVA) together with the post-hoc tests was developed by the senior author (S.S.S.) in C++.

Measurement of diversity and similarity

Diversity indices

A host of diversity indices have been proposed to measure species diversity (Magurran, 2004). Shaukat and Khan (1979) have investigated statistical behaviour of some of the important diversity inidices. Indices of diversity provide a useful means for quantifying community diversity and have been instrumental in revealing the microorganism diversity and community structure such as that associated with the phylloplane (Thomas and Shattock, 1986; Natsch *et al*., 1997; Joshi, 2008). A wide variety of diversity indices have been employed to compare the phylloplane mycobiota inhabiting mangrove species at different sites. Various diversity measures estimate different aspect of community structure. The general species diversity of the fungal communities was measured by the popular Shannon–Wiener information theory function:

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

Where H' is the general species diversity and P_i the proportion of total number of CFUs/cm^2 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:

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

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:

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

Robust non-parametric estimates of species richness were obtained by two different techniques: 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:

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

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

1. Randomly draw n samples with replacement 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 jackknifing 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:

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

in which n_i equals the number of CFUs/ $cm^2$for a fungus species.

For the computation of diversity indices, their variances and the dominance concentration, a program package was developed by one of us (S.S.S.) in C++ (Shaukat and Siddiqui, 2005) and is available from the senior author at a nominal cost.

Measurement of Similarity:

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

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

Where S_{jk} is the similarity between sites j and k and a, b and c are the usual notations of the contingency table.

The program SIMIL for computation of similarity matrix using eight different similarity indices was developed by the first author (S.S.S.) in C++ (Shaukat and Siddiqui, 2005).

Table 1. Abundances (CFUs/ml) of fungal species recorded on leaf surface of *Ceriops tagal* and *Aegiceras corniculatum* at three localities each.

Species	***Ceriops tagal***			***Aegiceras corniculatum***		
	Sonmiani	Keti Bunder	Greenhouse	Korangi	Keti Bunder	Greenhouse
Aspergillus candidus	1	3	2	1	3	-
A. terreus	7	10	4	4	9	3
A. flavus	3	6	1	2	7	5
A. fumigatus	5	-	3	3	6	1
A. sulphureus	1	1	1	2	1	1
A. wantii	1	2	2	8	-	-
A. niger	12	15	18	6	5	9
Acremonium sp.	3	1	2	2	4	3
Alternaria alternata	4	2	5	-	1	5
Fusarium moniliforme	4	5	2	2	-	1
Drechslera australiensis	3	1	1	-	2	1
F. oxysporum	5	2	7	4	6	6
Rhizopus stolonifer	4	2	5	5	-	8
Cladosporium cladosporiodes	3	-	4	-	-	3
Cladosporium globosum	2	1	1	3	3	2
*Curvularia*sp.	1	5	-	6	5	1
Penicillium chrysogenum	1	6	3	2	5	2
Penicillium citrinum	2	7	2	3	-	1
*Gliocladium*sp.	3	-	1	-	-	-
Memnoniella sp.	2	1	-	-	2	-
Mucor hiemalis	4	5	1	3	2	1
Mycelia sterilia (white)	1	1	-	1	1	-
Mycelia sterilia (yellow)	1	2	-	-	1	-
Mycelia sterilia (red)	-	1	1	-	1	1
Trichoderma viride	2	2	4	7	2	2
Fusarium solani	1	4	6	5	1	4
Paecilomyces sp.	1	2	2	-	-	-
*Phoma*sp.	2	3	-	1	-	-
Ulocladium sp.	-	1	1	1	-	-
S (number of species)	27	26	24	21	20	20
Number of genera	16	16	14	11	12	11

RESULTS AND DISCUSSION

The composition of phylloplane fungal assemblages and the abundances in terms of average CFU/cm^2 for the two mangrove species *Ceriops tagal* and *Aegiceras corniculatum* at three localities each are presented in Table 1. The total number of fungal species varied with the assemblage. On an overall basis twenty- nine microfungal species and 17 genera were recorded. The highest numbers of fungal species (27) and genera (16) were recorded at Sonmiani on the phylloplane of *Ceriops tagal*. The phylloplane mycobiota assemblages showed greater number of species and genera for *Ceriops tagal* compared to that of *Aegiceras corniculatum.* On the other hand, the lowest

number of species (20) were recorded from *Aegiceras corniculatum* growing at Keti Bunder. Phylloplane mycobiota derived from glasshouse collection of mangrove species showed lesser number of species compared to those of naturally growing mangrove plants.

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 fungal species on the phylloplanes of *Ceriops tagal* and *Aegiceras corniculatum*.

Diversity measures	*Ceriops tagal*			*Aegiceras corniculatum*		
	Sonmiani	Keti-Bunder	Green-house	Korangi	Keti-Bunder	Green-house
Species diversity (*H*')	3.038	2.933	2.799	2.867	2.761	2.713
Variance of diversity var(*H*')	0.008	0.008	0.012	0.006	0.008	0.001
Equitability (*J*')	0.921	0.900	0.880	0.942	0.921	0.905
Variance of Equitability var(*J*')	.0007	.0007	0.001	0.0006	0.0008	0.001
Species richness (d)	3.037	2.710	2.710	2.492	2.443	2.581
Dominance (D)	0.049	.059	0.077	0.059	0.066	0.066
Total # of species ($\hat{S}_{jack}$)	34.3	33.8	31.2	27.5	26.7	26.3
($\hat{S}_{boot}$)	32.7	31.3	29.8	25.8	24.9	25.7

Table 3.Similarity between fungal assemblages on phylloplane of *Ceriops tagal* and *Aegiceras corniculatum* at three localities for each mangrove species. Greenhouse was located at the Institute of Environmental Studies, University of Karachi, Karachi.

Species/Sites	*Ceriops tagal*			*Aegiceras corniculatum*		
	Sonmiani	Keti Bunder	Green-house	Korangi	Keti Bunder	Green-house
C. tagal: Sonmiani	X	82.7	75.8	67.8	71.4	67.8
C .tagal: Keti Bunder	_	X	72.4	70.4	74.1	64.3
C. tagal: Greenhouse	_	_	X	62.9	60.7	76.0
A. corniculatum: Korangi	_	_		X	57.7	60.0
A. corniculatum: Keti Bunder					X	70.8
A. corniculatum: Greenhouse						X

Generally, the phylloplane mycobiota was predominated by the genus *Aspergillus.* Particularly, *A. niger*, *A. terreus* and *A. flavus* were found to be the most abundant species. ANOVA for *Aspergillus niger,* which was a major species, showed significant difference in abundance with respect to the two mangrove species ($P<0.01$) and sites ($P<0.05$). The abundance of *Aspergillus terreus* exhibited significant difference with regard to mangrove species ($p<0.05$) but did not differ significantly with respect to sites. *A. flavus* did not show significant difference with regard to mangrove species but exhibited significant effect of site ($P<0.05$). Moreover, on an overall generic level, *Aspergillus* with regard to its abundance showed significant effect of both mangrove species and site (for both $p<0.05$). The present study accords well with the findings of Mehdi and Saifullah (1992) who also reported high abundance of *Aspergillus* species (i.e., *A. niger* and *A. flavus*) on the phylloplane of grey mangrove *Avicennia marina* growing at Clifton and Korangi Creek. Naikwade *et al*., (2012) recorded 9 different species of *Aspergillus* from the phylloplane of a mangrove species (*Ceriops tagal*); they also found *Alternaria alternata* on the phylloplane. The phylloplane microfungal assemblages for the greenhouse populations of both the mangrove species showed relatively lower number of genera as well as species. Shaukat *et al*. (2013) working with phylloplane mycobiota of *Avicennia marina* and *Rhizophora mucronata*, also observed dominance of *Aspergillus* species. Kuthubutheen (1981) in an extensive study involving 9 mangrove species, reported various fungal species included

in the genera like *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium* and *Trichoderma* which have also been reported in the current 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 noticed. These results correspond well with those of earlier workers with regard to phylloplane mycobiota of mangrove species. When diversity of fungal assemblages were measured, it was found that the general diversity (H') was slightly but consistently higher for *Ceriops tagal* compared to *Aegiceras corniculatum* (Table 2). However, equitability component of diversity (J') was slightly higher for *Ceriops tagal* compared to that of *Aegiceras corniculatum*. The greater fungal diversity for the phylloplane of *Ceriops tagal* could be attributed to its greater leaf size. It has been shown that size of resource unit affects the number of species that can co-occur (Sanders and Anderson, 1979; Barlocher and Schweizer, 1983). This notion is further supported by an earlier study where greater diversity was found for *Rhizophora mucronata* (with larger leaf) compared to *Avicennia marina* (Shaukat *et al*., 2013). Variances of diversity and equitability were not unexpectedly constantly low for both species and the sites. Species richness (d) was consistently higher for *Ceriops tagal* compared to that of *Aegiceras corniculatum.* Jakknife ($\hat{S}_{jack}$) and bootstrap ($\hat{S}_{boot}$) estimates of total number of species of phylloplane assemblages were constantly and substantially higher than the actual number of observed fungal species as these include those rare species that could not be included in the sample. Species diversity may be important because of its possible role on the establishment and coexistence of species (Nicholson, 1972) though in some model systems it is found to play hardly any role on these processes (Stohr and Dighton, 2004). Dominance concentration (D) was found to vary inversely with the general diversity (H') which is in agreement with the results of Shaukat *et al.* (1981). 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 (Lindow and Brandl, 2003). The qualitative similarity in the phylloplane mycobiota assemblages of the two mangrove species at three sites is represented in Table 3. Interestingly, the fungal assemblages on *Ceriops tagal* phylloplane at the three different sites exhibited high degree of similarities (Table 3). Generally, similarities between the assemblages were high. Usually the different sites for the same species showed higher similarity compared to those of the different species. Phylloplane mycobiota from glasshouse populations generally showed relatively lower similarity with those of the natural populations but the glasshouse phylloplane mycobiota for both species exhibited markedly high order of similarity (76%), thereby suggesting the influence of the local environment associated with glasshouse populations which could be both due to locality factor (Karachi University Campus) as well as the specific conditions prevailing in the greenhouse. It is interesting to note that the dominant phylloplane fungal species belonging to *Aspergillus*, *Fusarium*, *Cladosporium*, etc were also the dominant species of airborne fungi (Afzal *et al*., 2005; Rao *et al*., 2009). Based on the degree of correspondence between phylloplane and airborne mycobiota, it seems that the phylloplane microfungal assemblages contribute a great deal to airborne mycobiota. Likewise, Levetin and Dorsey (2006) asserted that the leaf-surface fungi are the major contributors to the airborne mycobiota. Those taxa with an airborne dispersal are the major components 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. Fungi are essential for nutrient mobilization, storage and release during the process of decomposition of plant parts, e. g., leaves in an ecosystem (Kjeller and Struwe, 2002). The examination of mycobiota associated with leaves would help to gain greater understanding of the role of fungi in litter decomposition and eventually nutrient cycling in the mangrove ecosystem.

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