Phyllosphere Fungi of *Alnus nepalensis, Castanopsis hystrix* and *Schima walichii* in a Subtropical Forest of North East India

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Abstract: A total of 38 epiphytic and endophytic phyllosphere fungi were isolated from living leaves of *Alnus nepalensis, Castanopsis hystrix* and *Schima walichii* by using a combination of cultural methods i.e. dilution plating, washed disk and surface sterilization, respectively at bimonthly intervals during July, 2008 to May, 2009. *Alternaria alternata, Cladosporium cladosporioides, Fusarium oxysporum* and *Pestalotiopsis* sp. were the dominant colonizers of three forest tree leaves. The type of fungal species isolated from different test leaves were found to be influenced by the method of isolation. Some species could be recovered by a particular culture method while others were recovered by two or all three isolation methods. *Alternaria raphani*, *Epicoccum purpurascens* and *Gliocladium roseum* from *Alnus nepalensis* leaves and *Scopulariopsis* sp. and *Trichoderma harzianum* from *Castanopsis hystrix* were the species recovered specifically by washed disk method. Whereas, *Gliocladium fimbriatum* was isolated only from *Schima walichii* leaves as endophytic fungi. [Journal of American Science 2010:6(3):118-124]. (ISSN: 1545-1003).

Key words: Phyllosphere fungi, epiphytes, endophytes, *Alnus nepalensis, Castanopsis hystrix, Schima walichii*

1. Introduction

The phyllosphere is the living leaf as a whole and includes the surface (phylloplane) and internal tissues colonized by a variety of epiphytic and endophytic microorganisms respectively, thereby occupying two distinct habitats on the leaf (Andrews, 1996; Carroll *et al.,* 1977; Petrini, 1991). The interest shown in the last few years in the study of phyllosphere microbes is due principally to their interactions with plants, herbivores and pathogens on living leaves which may be involved in the plant immunity system, reabsorption of organic and mineral matters from leachates, redistribution of nutrients prior to leaf fall and participation in the primary degradation of plant tissues (Carroll *et al*., 1977; Cabral, 1985; Lindow and Brandl, 2003; Osono, 2006). Another aspect of colonization ecology of phylloplane and/or phyllosphere fungi principally relates to the prevailing microenvironmental conditions on the leaf surfaces and their physical, chemical and phenological properties which affect the fungal establishment thereon (Pandey, 1990; Dix and Webster, 1995).

Studies on endophytic fungi in tree leaves have been carried out for several host species, when their significance as common symbionts and possible mutualists of plants were recognized (Carroll, 1995). Majority of such studies describing the diversity of phyllosphere fungi on different hosts and in various habitats have however, dealt with either annuals or perennials bearing short-lived deciduous leaves. Less is known about the long-lived leaves of evergreens (Mishra and Dickinson, 1981) though Ruinen (1961) demonstrated that the persistent leaves of tropical plants supported complex and extensive microbial flora.

North eastern India forms an important portion of Indo-Burma biodiversity hot spots (Pawar *et al.*, 2007). The nature and abundance of epiphytic and endophytic leaf fungi have been studied mainly in cool and temperate forests, but their investigation in other regions of the world e.g. warm temperate to tropical or subtropical forests are less explored (Heredia, 1993; Hata *et al*., 2002). Moreover a perusal of available literature reveals that no attention has been paid to the phyllosphere mycoflora of *Alnus nepalensis, Castanopsis hystrix* and *Schima walichii* in subtropical habitats of North eastern India.

Therefore, with this perspective it was thought desirable to undertake a preliminary study on the diversity of phyllosphere fungal community of three dominant tree species in a natural mixed subtropical forest of Manipur, North eastern region of India.

2. Material and Methods

2.1. Collection of samples

The studies were conducted in a natural mixed forest located in the Taphou Naga hill range (25° 15′ N Latitude and 94° 15′ E Longitude) at an altitude of 1200 m asl, which is about 2 km north-west of Senapati District headquarter and 65 km north of Imphal city, Manipur, India. The mean minimum and maximum temperature during the study period (July 2008 – May 2009) ranged between 13.9 ^{o}C to 25.1 ^{o}C in the months of

January 09 and July 08, respectively. The mean relative humidity varied from 56.9% to 84.6% whereas the total monthly rainfall ranged between 0 mm to 210.2 mm (Fig. 1). Healthy mature green leaves of *Alnus nepalensis, Castanopsis hystrix* and *Schima walichii* were collected from each of 5 trees randomly from the branches at 10 ft height in the canopies, separately. Leaves from 5 branches of each tree species, representing both the margin and interior of the canopy were sampled at bimonthly intervals during July, September and November, 2008 and January, March and May, 2009, respectively. The leaves of each tree species were kept in separate polyethylene bags and brought to the laboratory for isolation of epiphytic and endophytic phyllosphere fungi.

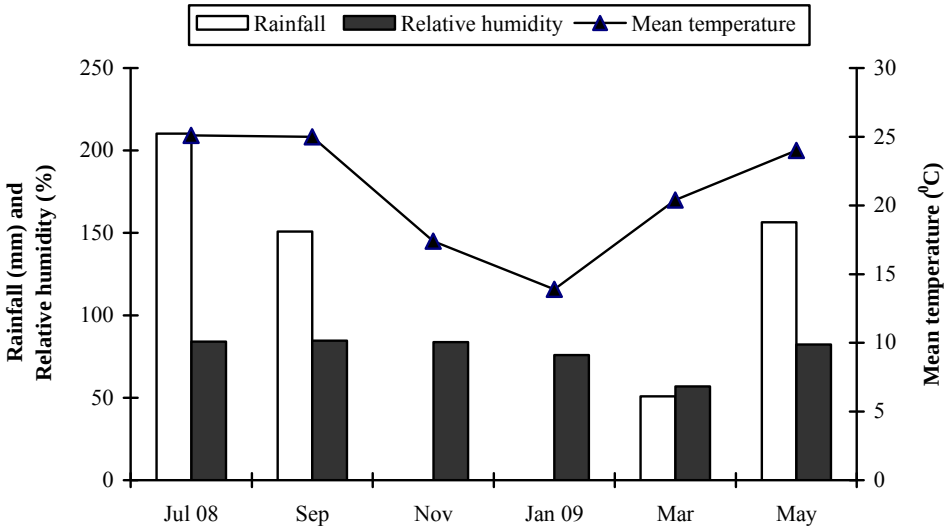

Figure 1. Monthly changes in climatic variables during the study period.

2.2. Isolation procedure

(a) Dilution plate method: One hundred leaf disks (5 mm diam.), two from each of fifty leaves of a tree species, were punched out with the flame sterilized cork borer and transferred separately, into 250 ml conical flasks containing 100 ml sterilized distilled water. The flasks were shaken for 20 min on a horizontal mechanical shaker to detach the fungal propagules present thereon. One ml aliquot of diluted suspension (10^{-3}) was pipetted out separately into each of 5 Petri dishes (9 cm diam) and 15 ml cooled (35 °C) and molten Czapek-Dox + 0.05% (w/v) Yeast extract agar medium supplemented with Streptomycin (100 mg/l). The plates were gently rotated clockwise and anticlockwise to ensure uniform distribution of homogenates and then incubated at 25 ± 1 °C under fluorescent light for 7 days. The fungal colonies appeared on the plates after incubation were identified. Isolates that remained sterile were recorded as sterile mycelia.

(b) Washed disk method: Twenty five disks (5 mm diam) of each leaf type were procured separately, and serially washed in 10 changes of sterile distilled water (1 min/washing). The disks were then dried in folds of sterile filter paper and five disks were inoculated at equal distance in each of 5 Petri plates containing 15 ml cooled Czapek–Dox agar medium. The plates were incubated as above.

(c) Surface sterilization method: Twenty five (5 mm diam) leaf disks of each tree species were prepared as above and submerged in 70% ethanol for 1 min, then transferred into 15% H_2O_2 for 1 min and again kept into 70% ethanol for 1 min (Kinkel and Andrews, 1988). Thereafter, the disks were serially washed in 10 changes of sterile distilled water, then blotted dry, inoculated in each of 5 Petri dishes (5 disks/plate) containing Czapek–Dox agar medium and incubated as above.

2.3. Calculation

The relative abundance (%) of each fungal species isolated by dilution plating was calculated as: (Number of colonies of a fungal species/ Total number of fungal colonies) × 100. Percent frequency of occurrence of each fungus recovered by washed disk and surface sterilization methods was calculated as: (Number of leaf disks on which a fungal species occurred/ Total number of leaf disks observed) × 100.

3. Results and Discussion

A total of 24 and 38 endophytic and epiphytic fungal species, respectively were isolated

from phyllosphere of *Alnus nepalensis, Castanopsis hystrix* and *Schima walichii* living leaves by using different cultural methods. Out of which 22 species were common to the three leaf types recovered at least by two or all employed methods (Tables 1, 2 & 3). *Alternaria alternata, Cladosporium cladosporioides, Fusarium oxysporum* and *Pestalotiopsis* sp. were the dominant surface and interior colonizers of different tree species leaves. In general, these species were extensively reported as common primary saprobes and ubiquitous hyphomycetes from attached leaf surfaces of wide variety of plants throughout the world (Breeze and Dix, 1981; Mishra and Dickinson, 1981; Pandey, 1990; Andrews, 1996; Osono, 2006) which can withstand on adverse conditions such as desiccation, UV radiation and microbial lysis by producing thick walled pigmented multicellular spores and microsclerotia (Hudson, 1968; Sadaka and Ponge, 2003). These fungi are normally encountered as epiphytes, but some can also occur as endophytes (Petrini, 1991).

Table 1. Epiphytic fungi of three forest tree leaves isolated by dilution plate method.

Fungal species	Forest trees species																	
	Alnus nepalensis						*Castanopsis hystrix*						*Schima walichii*					
	Sampling months																	
	Jul	Sep	Nov	Jan	Mar	May	Jul	Sep	Nov	Jan	Mar	May	Jul	Sep	Nov	Jan	Mar	May
Alternaria alternata	16.7	15.2	-	-	-	20.0	19.4	-	-	-	-	15.4	27.3	-	-	-	-	-
Aspergillus flavus	23.5	-	-	-	-	-	-	16.7	-	-	-	-	-	-	-	-	-	-
A. niger	23.0	22.2	27.3	-	-	-	18.9	-	-	-	-	-	26.1	-	-	-	-	-
Cladosporium sp.	-	-	-	36.0	9.3	-	-	-	-	-	10.5	20.0	-	-	-	28.1	-	-
C.cladosporioides	-	37.5	17.9	-	51.1	25.0	-	-	-	25.4	26.0	36.0	-	-	-	-	11.4	33.3
Curvularia lunata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	22.2	-
Fusarium oxysporum	-	18.5	33.3	14	-	-	-	-	-	-	-	31.8	-	-	39.3	-	14.3	-
F. poae	-	-	-	-	-	-	16.2	10.3	17.6	-	15.4	-	-	-	-	-	-	-
Gliocladium penicillioides	-	-	-	-	-	-	28.6	17.1	25.0	-	-	-	27.3	25.9	-	-	-	-
Graphium penicillioides	-	-	-	-	16.0	-	-	-	-	-	-	-	-	-	-	-	-	-
Mucor hiemalis	-	-	-	18.5	-	-	-	-	-	-	-	-	-	-	28.7	-	8.0	-
Paecilomyces varioti	-	18.7	42.9	-	-	-	-	-	-	-	10.0	-	20.0	23.5	-	-	-	-
Penicillium sp.	-	-	-	-	-	-	28.6	23.4	23.5	12.7	-	-	-	-	-	-	-	-
P. expansum	-	-	-	-	40.0	28.5	-	-	-	-	-	-	-	-	-	-	-	-
P. diversum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11.8	22.2	-
P. glabrum	-	-	-	-	-	-	36.4	-	-	-	-	-	-	-	-	-	-	-
P. italicum	-	-	-	16.7	-	28.5	-	-	-	-	-	-	-	-	-	-	-	-
P. javanicum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	33.3	26.3	22.9	81.8
P. rubrum	23.6	-	-	-	-	-	29.7	-	-	-	19.0	-	16.4	-	-	-	-	-
Pestalotiopsis sp.	-	30.5	38.2	-	11.1	25.0	-	-	36.0	15.9	17.0	-	-	40.9	30.4	-	-	-
Trichoderma koningii	26.0	-	-	-	-	-	31.3	20.6	-	-	-	-	-	-	16.7	-	-	-
T. viride	-	-	-	10.0	-	-	-	15.4	34.8	-	-	-	-	-	25.2	-	31.6	-
Trichothecium roseum	-	-	-	20.4	29.2	-	-	-	-	31.7	-	-	-	-	-	17.5	-	-
Verticillium terrestre	29.4	15.3	-	-	-	-	-	23.5	-	17.9	-	-	-	-	-	-	13.5	-
Dark sterile mycelia	-	-	-	-	13.3	50.0	-	-	-	17.6	20.0	16.6	-	-	-	25.0	30.0	-
White sterile mycelia	-	-	-	-	-	40.0	-	11.1	-	-	17.5	23.0	-	20.9	-	15.8	-	-

Table 2. Epiphytic fungi of living leaves of three different forest tree species isolated by washed disk method.

Fungal species	Forest trees species																	
	Alnus nepalensis						*Castanopsis hystrix*						*Schima walichii*					
	Sampling months																	
	Jul	Sep	Nov	Jan	Mar	May	Jul	Sep	Nov	Jan	Mar	May	Jul	Sep	Nov	Jan	Mar	May
Alternaria alternata	26.6	-	-	-	12.0	26.0	-	26.6	-	-	-	-	20.0	-	-	-	-	-
A. raphani	-	-	-	-	4.0	24.0	-	-	-	-	-	-	-	-	-	-	-	-
A. tenuissima	-	-	-	-	-	-	-	-	-	-	-	8.0	-	-	-	-	4.0	36.0
Aspergillus flavus	-	-	-	-	-	-	-	-	4.0	16.0	8.0	-	-	-	-	-	-	-
A. niger	20.0	20.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aureobasidium pullulans	-	-	-	8.0	8.0	-	8.0	8.0	20.0	-	-	-	-	-	-	-	-	-
Cladosporium cladosporioides	-	-	-	16.0	16.0	24.0	-	-	-	-	-	-	-	-	33.3		8.0	20.0
Epicoccum purpurascens	-	-	-	-	4.0	8.0	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium equiseti	-	-	-	-	-	4.0	-	-	-	-	-	-	-	-	-	-	-	-
F. oxysporum	13.0	-	33.3	12.0	12.0	-	-	6.6	6.6	-	-	20.0	40.0		6.6	12.0	4.0	-
Gliocladium penicillioides	-	20.0	-	-	-	-	6.6	-	-	-	-	-	-	6.6	-	-	-	-
G. roseum	-	-	-	-	4.0	8.0	-	-	-	-	-	-	-	-	-	-	-	-
Mucor hiemalis	20.0	20.0	6.6	60.0	-	-	-	-	-	-	-	-	-	6.6	20.0	-	-	-
Nigrospora sphaerica	-	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Paecelomyces varioti	33.3	-	-	-	-	-	-	-	-	-	-	-	-	6.6	-	-	-	-
Penicillium italicum	-	-	-	4.0	-	-	-	-	-	4.0	12.0	10.0	-	8.0	-	-	-	-
P. javanicum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	28.0	-	12.0	-
P. rubrum	-	-	-	-	-	-	-	-	-	-	-	-	20.0	-	-	-	-	-
Pestalotiopsis sp.	-	60.0	26.6	-	28.0	56.0	-	-	60.0	32.0	96.0	76.0	-	53.3	60.0	20.0	68.0	16.0
Scopulariopsis sp.	-	-	-	-	-	-	-	-	-	-	4.0	12.0	-	-	-	-	-	-
Trichoderma harzianum	-	-	-	-	-	-	-	-	-	-	8.0	4.0	-	-	-	-	-	-
T. koningii	20.0	-	33.3	-	-	-	20.0	33.3	-	-	-	-	-	-	-	-	-	-
T. viride	-	-	6.6	-	-	-	-	33.3	13.3	-	-	-	-	-	-	-	-	-
Trichothecium roseum	-	-	-	12.0	12.0	-	-	-	-	48.0	-	-	-	-	-	16.0	-	-
Verticillium terrestre	-	13.3	-	4.0	8.0	-	-	-	-	-	-	-	-	13.3	-	-	-	-
Dark sterile mycelia	20.0	20.0	-	4.0	4.0	-	-	-	-	-	-	-	-	-	-	-	-	-
White sterile mycelia	-	-	6.6	8.0	4.0	-	6.6	-	-	-	12.0		-	6.6	-	-	4.0	-

Table 3. Endophytic phyllosphere fungi of different forest trees isolated by surface sterilization method.

Fungal species	Forest trees species																	
	Alnus nepalensis						*Castanopsis hystrix*						*Schima walichii*					
	Sampling months																	
	Jul	Sep	Nov	Jan	Mar	May	Jul	Sep	Nov	Jan	Mar	May	Jul	Sep	Nov	Jan	Mar	May
Alternaria alternata	20.0	-	13.3	-	-	4.0	20.0	-	-	4.0	-	-	6.6	-	-	-	-	-
Aspergillus flavus	-	-	-	-	-	-	-	-	-	4.0	8.0	-	-	-	-	-	-	-
A. niger	-	46.0	-	-	-	-	20.0	-	-	-	-	-	26.6	-	-	-	-	-
Aureobasidium pullulans	-	-	-	12.0	-	-	-	-	-	-	-	-	-	-	-	24.0	-	-
Cladosporium cladosporioides	-	-	33.3	20.0	4.0	-	-	-	6.6	-	-	8.0	-	6.6	26.6	16.0	-	16.0
Curvularia pallescens	13.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium oxysporum	-	-	-	-	-	-	-	6.6	-	-	-	-	-	-	-	-	-	-
Gliocladium fimbriatum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13.3	16.0	16.0	-
G.penicillioides	-	-	-	-	-	-	20.0	13.3	-	-	-	-	6.6	13.3	-	-	-	-
Graphium penicillioides	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mucor hiemalis	-	-	-	-	16.0	-	26.6	6.6	-	-	-	-	-	-	-	4.0	-	-
Nigrospora sphaerica	-	-	-	-	-	4.0	-	-	-	-	-	24.0	-	-	-	-	-	-
Paecilomyces varioti	13.3	6.6	-	12.0	4.0	-	-	26.6	-	-	4.0	-	46.6	26.6	-	-	-	-
Penicillium expansum	-	-	-	-	16.0	16.0	-	-	-	-	-	-	-	-	-	-	-	-
P. italicum	-	-	-	28.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. rubrum	26.6	-	-	-	-	-	6.6	-	-	-	-	-	53.3	-	-	-	-	40.0
Pestalotiopsis sp.	-	33.3	33.3	-	12.0	-		20.0	6.6	4.0	16.0	52.0	-	-	33.3	-	-	-
Trichoderma koningii	-	-	6.6	-	-	-	-	-	-	-	-	-	-	-	-	32.0	24.0	-
T. viride	-	33.3	6.6	24.0	-	-	-	-	6.6	-	-	-	-	26.6	-	-	4.0	-
Trichothecium roseum	-	-	-	-	-	-	-	-	8.0	36.0	-	-	-	-	-	-	-	-
Verticillium terrestre	6.6	-	-	-	-	-	-	-	-	-	-	-	-	33.3	-	-	8.0	-
Dark sterile mycelia	-	-	13.3	-	64.0	-	-	-	6.6	20.0	8.0	-	-	-	-	-	-	4.0
White sterile mycelia	-	-	-	24.0	4.0	24.0	-	-	13.3	48.0	72.0	60.0	-	-	-	40.0	20.0	-
Unidentified I	-	-	-	-	-	16.0	-	-	-	-	8.0	-	-	-	-	-	-	-

Other fungi like *Aspergillus niger, Mucor hiemalis, Paecilomyces varioti, Penicillium rubrum, Trichothecium roseum, Trichoderma koningii* and *T. viride* were also found common on phyllosphere of at least two or all three leaves with varying relative abundance and/or occurrences (Tables 1, 2 & 3). Some of these species are able to utilize cellulosic components and gallic acid (Kjøller and Struwe, 1987; Rai *et al.,* 1988) and also found to play important role in primary degradation of plant tissues. In the present study, specific phyllosphere fungi showed differential seasonal preferences on three tree leaf types during various isolation periods. For example, *Gliocladium penicillioides* and *P. varioti* were recovered from *Schima walichii* leaves by dilution plating during rainy sampling months, whereas they were isolated during rainy and winter sampling periods from other test leaves. The variations observed in species richness and compositions of phyllosphere mycoflora on different leaf types during various sampling months can be assumed as the differences in competitive abilities, life cycle characteristics, potentialities to utilize residual organic chemical resources between the species present thereon (Osono, 2006). Besides these, prevailing environmental variables such as temperature, moisture and humidity during different sampling periods have also been reported

to affect the changes in population of specific phyllosphere fungi (Breeze and Dix, 1981).

The type of fungal species isolated from different test leaves were found to be influenced by the method of isolation. Some species could be recovered by a particular culture method while others were isolated by two or all three isolation methods. *Alternaria raphani, Epicoccum purpurascens* and *Gliocladium roseum* from *Alnus nepalensis* leaves and *Scopulariopsis* sp and *Trichoderma harzianum* from *Castanopsis hystrix* were the fungus occurred specifically by washed disk method whereas, *Gliocladium fimbriatum* was isolated only from *Schima walichii* leaves as endophytic fungi. *Fusarium equiseti* from *Alnus nepalensis, Penicillium glabrum* from *Castanopsis hystrix* and *Curvularia lunata* from *Schima walichii* were found only once during the sampling period which represent the specialization in a relatively narrow niche dimension by these fungi (Wildman and Parkinson, 1979). However, the possibility of a chance occurrence of certain fungal species on a particular leaf type cannot be overruled. Frequently recovered fungal species like *Aspergillus, Penicillium* and *Trichoderma* spp. from three plant leaf samples, grow quickly and produce large number of conidia which are easily dispersed and exhibit wide ecological spectrum (Christensen, 1981). The white and dark coloured sterile mycelia isolated from different leaf types (Tables 1, 2 & 3) must be representing those species which do not produce spores naturally or under cultural conditions including monokaryotic Basidiomycetes.

In conclusion, the present study revealed that despite the variation in physical, chemical and phenological properties in three leaf types, the fungal species isolated were more or less, similar and common to all except for some host and culture method-specific fungi. Further investigations on the dynamics of endophytic and epiphytic fungal species compositions associated with the same host leaves in other sites or during different seasons and increased sampling efforts could yield more fungal taxa and could further clarify the effect of host leaf on the fungal populations.

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