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RESEARCH ARTICLE

FUNGAL ENDOPHYTES ASSOCIATED WITH *NEPENTHES* KHASIANA HOOK.F., AN ENDEMIC PLANT OF MEGHALAYA, INDIA

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ABSTRACT

The paper highlights on the fungal endophytes associated with the insectivorous plant, *Nepenthes khasiana* of West Jaintia Hills of Meghalaya, India. Endophytic fungi were isolated from fresh roots, stem, leaves as well as pitcher cups. Out of the total isolates the mycelia sterilia and genus *Fusarium* represented the maximum number of species. *Acremoniumcerealis, Cladosporiumcladosporioides, Humicolagrisea, Phomaeupyrena* and mycelia sterilia are the most frequently occurring isolated from all the parts of the pitcher plant throughout the sampling period. Colonization frequency was highest in the Pitcher cup whereas the least in the stem. Shannon-Weaver diversity index (H') was highest in leaves (2.43)and lowest in roots(2.38) and *vice-versa* for Simpson's diversity index which indicates higher diversity in the leaves as compare to the other parts of the plant.

Key words: *Nepenthes khasiana*, Endophytes, Colonization frequency, Shannon-Weaver diversity index, Simpson's index of dominance, Environment.

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INTRODUCTION

Fungal endophytes are a group of fungi that during any of their growth phases asymptomatically colonize the internal tissue or organ of a plant (Arnold and Lutzoni, 2007). They establish their existence ubiquitously in various flora; from mosses and ferns to various gymnosperms and angiosperms (Arnold and Lutzoni, 2007; U"Ren *et al.,* 2012), including aquatic plants (Li *et al.,* , 2010). Their ability to co-exist and co-evolve with host plants is attributed to the balance accomplished between the virulence of endophytes and the host fortifications (Schulz *et al.,* 2006). Thus, the interaction between plant and endophytic fungi can be deliberated as a sort of symbiotic relationship. By inhabiting within the tissue of host plant fungal endophytes may acquire shelter and required nutrients whereas the plant may get aid from fungal endophytes over improved drought and flooding tolerance, amplified in the rate of biomass production and confrontation against harmful herbivore and pathogens. It is also believed that depending on the physiological state of the host plant, endophytes can live in plant either as mutualists or antagonists. These endophytes protect their hosts from infectious agents and confrontational conditions by secreting bioactive secondary metabolites (Azevedo, 2000; Carroll and Carroll, 1978;Strobel and Daisy, 2003). In recent years fungal endophytes have been reported to contribute significantly in the growth and defense mechanism and can also be attributed to the vast range of valuable compound produced such as enzymes and secondary metabolites (Fang *et al.,* 2005). Apart from enzymes, different types of secondary metabolites which have antibacterial, anti-inflammatory, anticancer, anti-tuberculosis and anti-malarial properties have also been identified. *Nepenthes khasiana*, an endemic threatened plant of Meghalaya, the only representative of the genus *Nepenthes* from India, is found to be distributed in Khasi Hills, Jaintia Hills and Garo Hills at an altitude ranging from 1000-1500m asl (Mao and Kharbuli, 2002). The characteristic feature of *N.khasiana* is well exhibited by the modification of the leaves into a specialized cup-like pitfall structure to trap a wide range of insects in order to cope up with the nitrogen and energy deficiency of the soil as they grow in a very hostile environment (Kitching and Schofield, 1986; Mohan and Clarke, 2010). Digestion of prey within the pitcher cup is regulated by a number of enzymes such as proteases (Athauda *et al.,* 2004), lipases (Tokes *et al.,* 1974), ribonucleases (Stephenson and Hogan, 2006), and chitinases (Eilenberg *et al.,* 2006). The plant has some medicinal values. The people of Khasi, Garo and Jaintia hills are acquainted with the medicinal properties of pitcher plant.

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The extracted juice from leaves is said to be helpful for diabetic patients as well as for those who suffer from difficulties in passing urine. Khasi and Garo people use the fluid of unopened pitcher as an eye drop for cataract and night blindness. The Jaintias use *N.khasiana* for treating the inflamed skin, stomach trouble and gynecological problems (Mandal and Mukherjee, 2011). Apart from certain ethno-medicinal uses, Hypoglycemic and hypolipidemic effect of *N.khasiana* have been clinically tested in rats. Recently a team of researchers at Tel-Aviv University of Israel have found that the liquid inside the pitcher contains a compound that fights off fungal infection by breaking down the fungal chitinous cell wall (Eilenberg *et al.,* 2010). Hence, pitcher plants are worthy alternative host plants to isolate beneficial fungal endophytes. The present investigation is an attempt to provide an overview on the diversity of endophytic fungi from *N. khasiana*.

MATERIALS AND METHODS

Plant material collection: Healthy plants of *N. khasiana* were sampled and collected on a basis of monthly interval from West Jaintia Hills of Meghalaya, India (Fig. 1), during the period from October, 2014 to September, 2015. Fresh samples were brought to the laboratory and further processed within 24 hours of collection.

Description of Study site: The natural population of *N. khasiana* Hook.f. in the area of Amlarem village, West Jaintia Hills District, Meghalaya, India, located between 25^0 17' N Latitude and 92^0 03' E Longitude was selected for the present study. The climate is warm and temperate where the summers are much rainier than winters. The average annual temperature is 18.6 °C. In a year, the average rainfall is 4974 mm. October and November is the transition period (autumn) between rainy and winter seasons. The period between December and February is characterized by cold and dry weather conditions (Source- Agricultural department of Meghalaya).

Determination of basic soil properties: Soil temperature was recorded at the time of sampling collection with the help of soil thermometer. The moisture content was determined by oven dry basis by drying a known amount of freshly collected soil sample in a hot air oven at 105°C for 24 hours and reweighing the dried sample till a constant weight was obtained. The percentage moisture content was calculated as follows: Moisture content (%) = (W1xW2)/ (W1) x100; Where, W1=initial weight, W2=final weight after 24 hours. Soil pH was determined electrometrically in soil-water suspension at the ratio of 1:2.5. Ten grams of freshly collected soil was taken in a beaker having 50ml of distilled water and stirred for 15 minutes on a magnetic stirrer. The solution was then kept overnight and the pH was recorded using electronic digital pH meter.

Isolation and identification of the endophytic fungi: The samples were surface sterilized following the slightly modified protocol of Bayman *et al.,* 1997. The samples were washed under running tap water and cut into pieces of 0.5 cm diameter. These segments were then surface sterilized by immersing them in 75% ethanol for 1 minute, followed by treatment in 2% (v/v) sodium hypochlorite (NaOCl) for 3 minutes, then again immersing them in 75% ethanol for 30 seconds and then lastly rinsed for three times with sterile distilled water to remove any traces of surface sterilants. The excess moisture adhering to the processed segments were removed by blotting with sterilized Whatman No.1 filter paper. The samples thus prepared were then inoculated on to a Petri dish containing PDA (Potato Dextrose Agar) medium amended with Streptomycin (200mg/l). The Petri dishes were sealed using Parafilm^{TM} and incubated at 25 ± 1^oC in an incubator which was monitored every day for the growth of endophytic fungi. Further to the development of colonial growth on the medium, the fungi were isolated from the main culture plate and subsequently cultured to raise pure colonies. The isolated endophytic fungi were identified to genus or species level based on their macro and microscopic morphology andby comparing standard monographs by Subramaniam (1971), Barnett and Hunter (1972) and Domsch *et al.,* . (1980).

Statistical analysis: The colonization frequency (%CF) of endophytic fungi was calculated and determined by using the formula given by (Hata and Futai, 1995) :CF (%) = $(N_{Col} /N_t) \times 100$,Where, N_{Col} is the number of segments of plant tissue colonized by each fungus and N_t is the total number of segments of plant tissue studied. The fungal diversity of endophytic population was estimated with the following diversity indices. The reason for using these diversity indices was to take advantage of the strength of each index and to predict the complete structure of the population. Simpson's index (D) was calculated by following formula(Simpson, 1951): $D = \sum(n/N)^2$, Where, n represents the total number of isolates of a particular species and N is the total number of isolates of all species. Simpson's index of diversity = 1-D, Where, D is Simpson's index. Species Richness Species richness is a measure of the number of species found in a sample. This particular measure of species richness is known the Menhinick's index: Species richness = $S/\sqrt{N}$, Where, S is the total number of species. Index of general diversity (H') or Shannon and Weaver (1949) diversity (Shannon and Weaver, 1949): Shannon Index (H') = $-\Sigma pi \ln pi$, Where, pi = n/N, n is the total number of isolates of a particular species, N is the total number of isolates of all species and ln is the Natural Log. Pielou's evenness J' (Pielou 1995), which is expressed by the Shannon information scaled by the maximum information, to measure species evenness for each community: J' = H'/ln(S), Where, H' represents the observed value of Shannon index and S is the total number of species observed. All the statistical analyses were achieved with the software PAST3 and MS Excel.

RESULTS AND DISCUSSION

A total of 26endophytic fungi were isolated and identified from different parts of N. khasiana representing 18 genera viz. *Acremonium sp., Cladosporium sp., Colletotrichum sp., Cylindrocladium sp., Fusarium sp., Humicola sp.,Gonytrichum sp., Mortierella sp., Nectria sp., Nigrospora sp.,Oidiodendron sp., Talaromyces sp., Phomasp., Phytophthora sp., Pythium sp., Scytalidium sp., and3mycelia sterilia.* The group of mycelia sterilia consists of several morphological fungal varieties, but then again not forming true spores. This group of fungi is significantly predominant in endophyte studies (Lacap *et al.,* 2003). The fungal isolates were mainly composed of Ascomycota (13 genera; 18 species), followed by Oomycota (2 genus; 3 species) and Zygomycota (1genus; 2 species) (Table 1). High percentage of ascomycota as endophytic fungi were also reported by the work of Goveas *et al.,* (2011) from Cosciniumfenestratum- a red list endangered medicinal plant, it could be due to the ability of Ascomycota to produces

Table 1. Percentage Frequency (%CF) of endophyticfungi in different parts of the plant

Sl. No.	Genera	Endophytic fungi	%CF				Total %CF
			Leaves	Stem	Roots	PC	
1	Ascomycota	*Acremoniumcerealis* (P. Karst.) W. Gams 1971)	-	18.1	6.2	17.5	41.8
2		*A.murorum* (Corda) W.Games 1971)	13.5	-	4.1	-	17.6
3		*Cladosporiumcladosporioides* (Fresen.) G.A. de Vries 1952)	2.7	11.3	12.5	-	26.5
4		*C.macrocarpum* (Preuss 1848)	-	-	-	5	5
5		*Colletotrichumgloeosporioides* (Penz.) Penz. & Sacc.1884)	8.1	-	-	10	18.1
6		*Cylindrocladiumscoparium* (Morgan 1892)	-	6.9	-	-	6.9
7		*Fusariumaquaeductuum* (Radlk & Rabenh.) Lagerh. & Rabenh. 1891)	2.7	-	-	-	2.7
8		*F. oxysporum* (Schltdl. 1824)	-	-	-	5	5
9		*F.sporotrichioides* (Sherb. 1915)	5.4	2.2	-	-	7.6
10		*Gonytrichummacrocladum* (Sacc.) S. Hughes 1952)	-	2.2	-	-	2.2
11		*Humicolafuscoatra* (Traaen 1914)	5.4	4.5	4.1	-	14
12		*H. grisea* (Traaen 1914)	8.1	6.8	10.4	-	25.3
13		*Rectifusariumventricosum* (Appel & Wollenw.) L. Lombard & Crous, in Lombard, van de Merwe, *Groenewald & Crous* 2015)	-	2.2	-	-	2.2
14		*Nigrosporaoryzae* (Berk & Broome)Petch 1924)	-	4.5	-	-	4.5
15		*Oidiodendron echinodermata* (G.L. Barron 1962)	-	-	8.3	-	8.3
16		*Talaromycespurpurogenus* (Samson, N.Yilmaz, Houbraken, Spierenb, Seifert, Peterson, Varga & Frisvard 2011)	-	13.6	14.5	15	43.1
17		*Juxtiphomaeupyrena* (Sacc.)Valenz.-Lopez, Crous, Stechigel, Guarro&J.F.Cano. 2017)	18.9	11.3	12.5	10	52.7
18		*Scytalidiumlignicola* (Pesante 1957)	2.7	-	-	-	2.7
19	Oomycota	*Phytophthoracinnamomi* (Rands 1922)	13.5	-	-	10	23.5
20		*Pythiumaphanidermatum* (Edson) Fitzp. 1923)	2.7	-	4.1	-	6.8
21		*Globisporangium irregular* (Busiman) Uzuharshi, Tojo & Kakish. 2010.)	-	4.5	10.4	2.5	17.4
22	Zygomycota	*Mortierellagamsii* (Milko 1974)	2.7	-	-	5	7.7
23		*Mortierella sp.*	-	-	4.1	5	9.1
24	Mycelia sterilia	MS(brown)	-	2.2	8.3	5	15.5
25		MS(yellow)	5.4	9.1	-	5	19.5
26		MS(white)	8.1	-	-	5	13.1

Table 2. Variation values of temperature, moisture content and pH of the soil sample

Sl.no	Month of collection	Soil Temperature (ºC)	Soil Moisture content (%)	Soil pH
1	October	24.9	57.6	5.78
2	November	21.6	56.2	5.77
3	December	17.2	54.2	6.74
4	January	14.18	53.7	6.66
5	February	14.39	58.3	6.65
6	March	18.8	78.2	6.32
7	April	19.39	83.6	6.45
8	May	21.17	83.8	6.23
9	June	21.66	84.1	6.18
10	July	22.38	79.1	5.81
11	August	24	88.7	5.79
12	September	25.15	88.0	5.80

ascospores which helps them to striveagainst other microorganisms through the harsh environmental circumstances. Soil temperature showed variation throughout the sampling period (Table 2). It was recorded to be the highest in September (25.15 ºC) and least in January (14.18 ºC). The pH of the soil was moderately to slightly acidic in nature which ranged from 5.77-6.66 (Table 2).pH of 6.66 was highest in January and lowest in November with a pH of 5.77. The moisture content of the soil ranged from 88.7 % to 53.7 %. It was highest in August (88.7 %) and lowest in January (53.7 %) (Table 2). It can be observed that flattering soil physico-chemical properties for the growth of fungal endophytesfalls under the months of August to January. Colonization frequency (%CF) of *Phomaeupyrena* (52.7%) was highest in all the plant sample, followed by *Talaromyces purpurogenum* and *Acremoniumcerealis* with % CF of 43.1 and 41.8 respectively (Table 1).These were the dominant genera or order of endophytic fungi similar to the findings reported in tropical endophytic fungi (Corrado and Rodrigues, 2004; Krohn *et al.,* . 2007). The Shannon diversity index (H') was highest in leaves (2.43) and lowest in roots (2.38), which indicates the *vise-versa* result for simpson's index with the value of 0.89 and 0.90 (Table 3). Species richness was highest in leaves (2.36) and lowest in roots (1.73).

Table 3: Shannon index (H') and Simpson's diversity index for leaves, roots, stem and pitcher cup

Sl.no.	Plant part	Shannon-Wiener index (H′)	Simpson's diversity index
1	Leaves	2.43	0.894
2	Stem	2.42	0.896
3	Roots	2.38	0.901
4	Pitcher cup	2.42	0.898

Fig. 1. Showing *N. Khasianaat* natural habitat

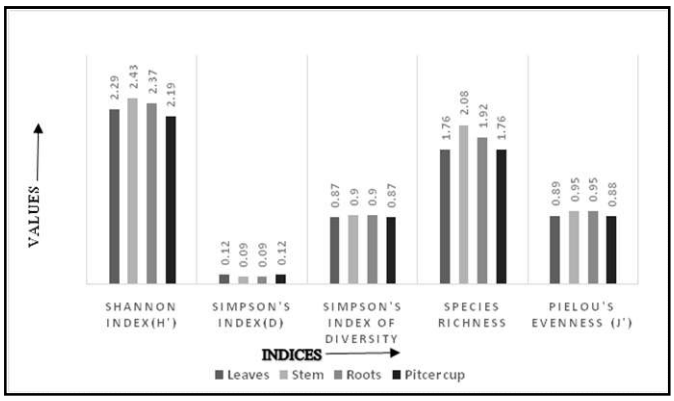
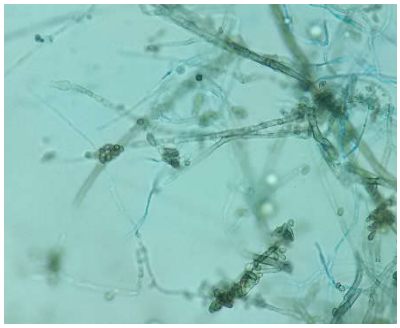
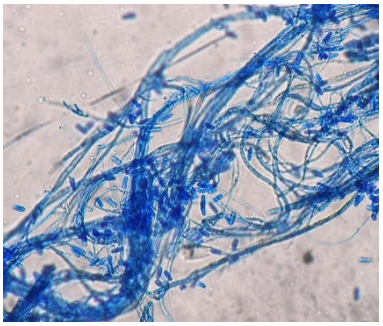


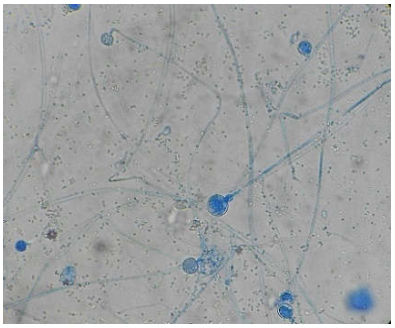
Fig. 2. Bar Diagram showing different diversity indices



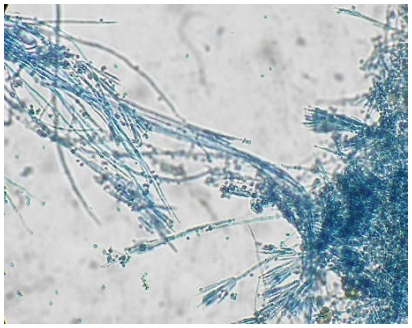
a) Cladosporium cladosporioides



b) Colletotrichum gloeosporioides

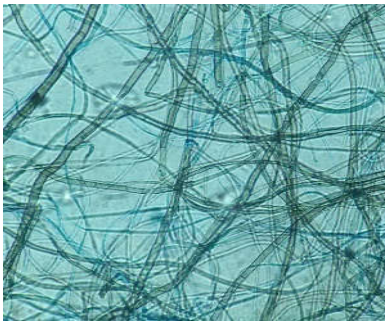


e) Mortierella sp.



f) Penicillium purpurogenum

g) Phoma eupyrena



j) Mycelia sterilia

Fig. 3. Microscopic diagrams of few endophytic fungi isolated from different plant parts of *N. khasiana*

Peilou's evenness (J') was highest in roots (0.96) and lowest in stem (0.91) (Fig. 2). The difference in endophyte assemblages from various tissues indicated that some fungal endophytes have an affinity for different tissue types and this might be a reflection of their capacity for utilizing or surviving within a specific substrate (different tissue texture and chemistry) (Rodrigues, 1994; Photita *et al.,* 2001). Many previous reports also discovered tissue-specificity in endophytic fungi (e.g., Taylor *et al.,* 2001; Ganley and Newcombe, 2006).Further studies are needed to reveal the interaction between the host plant and its endophytes.

Conclusion

Our results have showed that there are varieties of endophytic fungi colonizing *N. khasiana* an endemic plant of Meghalaya, India. Though isolation of endophytes has been accomplished from various forest types and locations around the globe, each study is unique in documenting newer endophytic taxa. With a view of investigating the diversity of endophytic fungi occurring in the plant tissues of *N. khasiana* were subjected to standard procedures for isolation of fungi. A total no. of 26 fungal endophytes was identified up to their species/genus level. Fungal Endophytes such as *Acremoniumcerealis*, *Colletotrichumgloeosporioides*, *Talaromyces purpurogenum*, *Phomaeupyrena* and mycelia sterilia were found as frequently occurring with high colonization frequency (%) within the plant. Thus the present study could be considered as an earnest attempt in exploring the diversity of fungalendophytes associated with pitcher plant.

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