



Endophytic fungal diversity of endemic carnivorous plant *Nepenthes khasiana* in Meghalaya, India

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Abstract

The present investigation deals with the isolation of endophytic fungi from leaf, stem, root and pitcher cup tissue segments of the endemic carnivorous plant *Nepenthes khasiana* collected from its natural habitat for a period of one year at monthly intervals. Out of 576 tissue segments inoculated, a total of 39 fungal endophytes along with mycelia sterilia were isolated from the host plant. To assess the diversity of fungal endophytes, the colonization frequency (%CF) was first recorded using past software and MS excel. The fungal isolates were mainly composed of the phylum Ascomycota, followed by Zygomycota and Oomycota. The highest percentage colonization frequency on an average of three replicates were recorded in pitcher cup tissues followed by root, stem and least was recorded in leaf of the host plant. Among the isolates, *Globisporangium irregulare* (83.33%) showed high % CF in leaf, *Juxtiphoma eupyrena* (83.33%) reported to have maximum % CF in the stem, *Talaromyces ruber* (66.66%) was recorded high % CF in root and mycelia sterilia (white) were showed the highest % CF in the segments of leaf. The diversity index analyses of Shannon-Weiner, Simpson's index, species richness and species evenness of diversity showed that leaf of *N. Khasiana* has the highest diversity than the other parts of the plant. So, with the help of the present finding, we conclude that the distribution of fungal endophytes and their % colonization frequencies vary within different tissues of the host plant and thus, this confirms tissue specificity nature of endophytic fungi.

Key words – Colonization frequency – Diversity indices – Natural habitat – Pitcher plant – Tissue specificity

Introduction

Many ancient literature indicate that synergistic collaboration among fungi and plants have taken place more than 400 million years ago (Krings et al. 2007). Morphologically fungi exist as both microscopic and macroscopic classes. As a result of its existence at the microscopic level, only a few numbers of fungi are recognized and depicted till now. The appellation “Endophytes” has been used in an extensive perception as per its description to comprise bacteria (Kobayashi & Palumbo 2000), fungi (Petrini 1991, Stone et al. 2000), actinomycetes (Bills et al. 2004, Stone et al. 2000, Tan et al. 2006), algae (Peters 1991) and some insects (Feller 1995) residing within the plant tissues deprived of any ostensible symptoms of the disease. Nearly all type of vascular plants are well-known to harbor endophytic microorganisms (Arnold et al. 2000, Sturz 2000). The ecological condition of the host also influences the endophytic scattering in a population (Hata et al. 1998). Endophytic fungi can enter the plant through its root and colonize different plant parts including

flowers and fruits as well, but through stomata or wounds in the plant many phyllosphere endophytes can also enter as those wounds or stomata acts as passage to such microorganisms (McCully 2001, Danhorn & Fuqua 2007, Schulz & Boyle 2006).

Nepenthes is the tropical carnivorous plant, has been known to have evolutionarily modified leaves as the peripheral digestive organs via leaf episcidation process (folding of the leaf towards the interior side with the ventral or upper surface flattening the inside of the pitcher) in order to receive nutrients from prey trapped inside the pitcher cups. For the digestion of the prey, *Nepenthes* reduces the pH of its fluid to facilitate the enzymatic reactions and to regulate the bacterial population present in the micro-habitat (Chan et al. 2016).

The present study aimed to isolate endophytic fungi from leaves, stems, roots and pitcher cups of an endemic plant of Meghalaya, *N. khasiana*. It is a carnivorous IUCN listed – endangered plant. *N. khasiana* is the only representative of the family Nepenthaceae from India and is considered to harbor vast range of medicinal properties used in traditional medicine by local people. Thus, the aspect of assessing the diversity of endophytic fungi will help to understand the association of fungal endophytes with the host plant.

Materials and methods

Sample collection

For the isolation of endophytic fungi, *N. khasiana* was selected as the host plant. The plants were randomly collected for a period of 1 year (2015-2016) at monthly intervals from the West Jaintia Hills district of Meghalaya. The plants were collected aseptically in sterilized polythene bags which were taken to the laboratory and processed within 24 hours of collection.

Surface sterilization

The samples were surface sterilized following the slightly modified protocol of Bayman et al. 1997. The plant parts were vigorously washed under running tap water to remove dust and soil particles. The protocol given by Suryanarayanan et al. 1998 (modified) was used for the isolation of endophytic fungi from the sterilized plant samples. Different plant parts (leaves, stems, roots and pitcher cups) of *N. khasiana* were chopped into small fragments of 0.5 cm diameters. These segments were then surface sterilized by immersing them in 70% ethanol for 1-3 minute, followed by treatment in 2% (v/v) sodium hypochlorite (NaOCl) for 3 minutes, then again immersing them in 70% ethanol for 30 seconds and then lastly rinsed with sterile distilled water to remove any traces of surface sterilants. The excess moisture adhering to the treated segments were eliminated by blotting with sterilized Whatman No.1 filter paper.

Efficiency of surface sterilization

The effectiveness of surface sterilization was performed by washing the surface sterilized samples with sterile water few times followed by transferring the samples in 5 ml of sterile water. The sterilized sample-water mixtures thus obtained were then stirred for 1 min. An aliquot of 0.5 ml water obtained above (after removing the plant parts) was then inoculated on Potato Dextrose Agar (PDA) medium and incubated at 27°C for 7 days. Plates which show negative result considered as successfully surface disinfected and the procedure thus used for the isolation of endophytic fungi (Schulz et al. 1993).

Isolation of endophytic fungi

For the isolation of endophytic fungi, samples were dried under laminar airflow to avoid contamination. Prepared samples were inoculated on to a Petri dish containing PDA (Potato Dextrose Agar) medium amended with Streptomycin (200mg/l) to suppress any bacterial growth. The Petri dishes were sealed using Parafilm™ and incubated at 25±2°C in an incubator for 7 to 15 days. Further to the development of colonies on culture plates were studied by isolating them and again sub-cultured as pure culture on PDA and Czapeck Dox Agar (CDA) media. For characterization of the morphology of fungal isolates, slides were prepared and stained with lacto-phenol blue reagent and examined under a bright field

microscope. Identification was done by referring standard monographs (Ellis 1976, Domsch et al. 1980, Barnett & Hunter 1972, Subramanian 1971) and with the help of their morphological characteristics such as growth pattern, hyphal characters, colour of colonies on the medium, surface texture, margin character, aerial mycelium, mechanism of spore production and characteristics of the spores. The isolated fungal endophytes have been deposited to the Microbial Repository Centre (MRC), IBSD, Manipur, India. Culture samples that did not produce any spores were categorized separately and given a code based on colour of mycelium and morphological feature of colony. Molecular characterization (ITS sequence of rDNA) was done to confirm the identification of such sterile fungal strains and the nucleotide sequences obtained in this study were deposited to the NCBI- GenBank database with following accession numbers - MH458928.1, MH458932.1, MH510306.1, MH458934.1 and MH458933.1. Confirmation of the presence of endophytic fungal spores and elongation of new hyphae in different tissue parts of the host plant was performed with the help of Scanning Electron Microscopy (SEM) (Fig. 2).

Data analysis

With the intention to estimate the diversity of endophytic fungi, the colonization frequency (%CF) of endophytic fungi was calculated and determined by using the formula given by Hata & Futai (1995). Dominant endophytes were calculated as percentage of colony frequency of an endophyte divided by the sum of the percentage of colony frequency of all endophytes x100 (Tayung & Jha 2006).

$$CF (\%) = (N_{Col} / N_t) \times 100$$

Where, N_{Col} = Number of segments of plant tissue colonized by each fungus and N_t = 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. All the statistical analyses were achieved with the software package PAST3 and MS Excel (Hammer et al. 2001) following diversity indices were calculated: (a) Simpson's Index. (b) Simpson's index of diversity. (c) Species richness. (d) Shannon-Weaver diversity index. (e) Evenness was calculated.

(a) Simpson's index (D) was calculated by following formula (Simpson 1951): $D = \sum (n/N)^2$

Where, n = Total number of isolates of a particular species and N = Total number of isolates of all species.

(b) Simpson's index of diversity = 1-D (D is Simpson's index)

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: (c) Species richness = S/\sqrt{N}
Where, S = Total number of species.

Index of general diversity (H') or Shannon & Weaver (1949) diversity (Shannon & Weaver 1949): (d) Shannon Index (H') = $-\sum p_i \ln p_i$

Where, $p_i = n/N$, n is the total number of isolates of a particular species, N = Total number of isolates of all species and ln = 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: (e) $J' = H' / \ln(S)$

Where, H' = Observed value of Shannon index and S = Total number of species observed.

Results

During the study period, a total of 576 plant segments (leaves, stems, roots and pitcher cups)

were plated for the isolation of endophytic fungi. 4 segments per plates were inoculated on PDA medium plates with three replicate each (Fig. 1) and to maintain pure culture, Czapeck dox agar (CDA) medium plates have been used. Highest numbers of fungal endophytes were isolated from the leaf of *N. khasiana* followed by pitcher cup, stem and root for the study period. SEM images also confirm the presence of fungal endophytes within the different plant tissues of the host plant *N. khasiana* (Fig. 2). The fungal isolates were mainly composed of Ascomycota (25 genera; 35 species), followed by Zygomycota (3 genera; 6 species) and Oomycota (3 genera; 5 species).

A total of 39 endophytic fungi were isolated and identifies from the different plant parts of *N. khasiana* during the one year of study period. Table 1 depicts the list of endophytic fungi isolated from *N. khasiana*. Among these isolates, it was observed that the phylum Ascomycota dominated the endophytic assemblage within the host plant. In our study we detected that 61.53% of isolates belonged to Ascomycota followed by Oomycota, Zygomycota and mycelia sterilia with 12.82 % each. The fungal assemblage was found to be dominated by the class Sordariomycetes (25.64%) followed by Eurotiomycetes (20.51%), Dothideomycetes (10.25%), and Incetraesedis and Mycelia sterilia (12.82% respectively), Mucoromycetes (7.69%), Mortierellomycetes (5.12%), Pezizomycetes and Leotiomycetes (2.56% each). Endophytic fungi with maximum average percentage of colonization frequency (%CF) were considered to be dominant. During the sampling period, *Juxtiphoma eupyrena* and *Talaromyces ruber* exhibited highest dominance in the leaf with 15% each followed by *Globisporangium irregulare* with 8.33%. The endophytic assemblage in the stem was dominated by Mycelia sterilia white (16.39%), *J. eupyrena* (13.66%) and *T. ruber* with 12.29%. The dominant root endophytes were MS brown (22.78%) and *T. ruber* (10%). Whereas, in pitcher cup it was dominant by MS white (17.5%) followed by *Colletotrichum gloeosporioides* and *Gliomastix cerealis* with 10% each.

We found that *Alternaria alternata*, *Fusarium proliferatum*, *Gongronella butleri* and *Mortierella* sp.1 were isolated only from the leafs, *Periconia macrospinoso*, *Phymatotrichopsis omnivore*, Mycelia sterilia (black) and Mycelia sterilia (Red) were specific only to the stem, in root *Globisporangium intermedium*, *Pythium aphanidermatum*, *Rhizopus microspores*, *Rhizopus* sp. and *Scytalidium lignicola* were restricted to the root tissues and *Cosmospora butyri*, *Mortierella gamsii*, *Mortierella* sp. 2, *Penicillium jensenii* and *Phytophthora cactorum* were isolated only from the pitcher cups. However, *Cladosporium cladosporioides*, *Gliomastix cerealis*, *Juxtiphoma eupyrena*, *Penicillium brevicompactum*, *Talaromyces purpureogenus*, *T. ruber*, *T. rugulosus*, MS (brown) and Mycelia sterilia (white) were found to be present in all the plant parts of the sampling plant.

In which, *Globisporangium irregulare* (83.33%), *Penicillium glabrum* (66.66%) and MS white (58.33%) showed highest percentage of colonization frequency in leaf (Fig. 3). It was found that endophyte *J. eupyrena* (83.33%) and *Fusarium redolens* (33.33%) showed maximum %CF in stem (Fig. 4), *Talaromyces ruber* (66.66%), *T. purpureogenus* and MS (white) with %CF 58.33% was maximum in root (Fig. 5). However, *C. gloeosporioides* and *G. cerealis* showed %CF 66.66 in pitcher cup (Fig. 6), where the highest %CF was recorded by MS (white) with 116.66%.

Shannon index (H') was found to be highest in the leaf with the value of 2.78 followed by pitcher cup with 2.76 whereas it was recorded to be the least in root with value of 2.68, which indicates the vise-versa result for Simpson's diversity index (1-D). Simpson's index of dominance (D) was found to be highest in root with value of 0.09 and d index was least in leaf (0.07). Species richness (S/\sqrt{N}) was recorded to be highest in leaf with the value of 2.34 and minimum in leaf with value of 1.64. As like other indices, Species evenness (E) was also recorded and it was found to be highest in leaf with value of 0.96 whereas, it was recorded to be least in root with value of 0.89 (Table 2).

Discussion

In the present investigation, the endophytic fungi isolated from *N. khasiana* were mainly composed of Ascomycota (25 genera; 35 species), followed by Zygomycota (3 genera; 6 species) and Oomycota (3 genera; 5 species). However, high percentage of ascomycota as endophytic fungi were also reported by the work of Goveas et al. (2011) from *Coscinium fenestratum*- a red list

endangered medicinal plant, it could be due to the ability of Ascomycota to produce ascospores which helps them to thrive against other microorganisms through the harsh environmental circumstances. A total of 5 mycelia sterilia were also isolated. The group of mycelia sterilia consists of several morphological fungal varieties, but then again not forming spores under laboratory conditions. This group of fungi is significantly predominant in endophytic studies (Lacap et al. 2003). Other studies on endophytic fungi are also reported the presence of sterile forms in their survey (Suryanarayanan et al. 1998, Rajagopal 1999, Maheswari 2011). Several phylloplane fungi such as *Alternaria*, *Aureobasidium*, *Cladosporium*, *Epicoccum* belonged to Hyphomycetes were isolated as endophytes from a wide range of plant species growing in different habitats (Bills 1996, Peláez et al. 1996). These phylloplane fungi are proficient of penetrating the outer layers of the leaf or may be confined in the substomatal cavities (Cabral et al. 1993). In our study we also have witnessed similar finding where some of the phylloplane fungi viz. *Alternaria*, *Cladosporium* etc. were isolated as fungal endophytes.

In the case of the present investigated plant the endophytic fungi isolated were *Gliomastix cerealis*, *Juxtiphoma eupyrena*, *Talaromyces purpureogenus*, MS (Brown) and MS (white) showed highest colonization frequency (%CF) as compared to other isolated of the plant. And among all, *J. eupyrena* was observed as commonly occurring endophytes in all the plant parts of *N. khasiana*. Similar occurrence of *J. eupyrena* was observed in the tissues of *Artemisia thuscula* by Cosoveanu et al. (2018). It was observed that in selected plant for present investigation, the Shannon diversity index (H') was higher in the aerial part of the plant i.e. in leaf followed by the underground part (root) and least was observed in stem of the plant, this is the opposite of the previous finding by Jin et al. (2013). However, Simpson's index of dominance (D) showed the vice-versa result. Species richness was also reported to be higher in leaf and lower in roots during the two year of the study period. A similar finding was reported by various researchers viz. Kumar & Hyde (2004), Raviraja (2005), Huang et al. (2008), Sun et al. (2008), Xing et al. (2010), Chaeprasert et al. (2010), Thalavaipandian et al. (2011), Siqueira et al. (2011) and Suwannarach et al. (2011). This could be due to the fact that *N. khasiana* is a carnivorous plant that grows in the soil where the nutritional value and pH of the soil is low and therefore, they adjust their requirements through the captured and digested prey. Moreover, the surface area of leaf is supplementary as compared to the roots of pitcher plants. The time of leaf exposure may also have accounted for the better density of endophytic fungi (Fröhlich et al. 2000, Toofanee & Dulyamode 2002, Arnold & Herre 2003). Huang et al. (2008) also pointed out the tissue specific trait of endophytic fungi although most of the endophytes only exhibited tissue preference this partially supports our finding where a higher number of endophytes has been isolated from a specific tissue as compared to other tissues of the host plant.

Table 1 List of fungal endophytes isolated from different plant parts (leaf, stem, root and pitcher cup) of *N. khasiana* during the study period of 2015-2016.

Sl. No.	Endophytic fungal isolates	L	S	R	PC
Phylum: Ascomycota- 17 Genera, 24 Species					
Class- Sordariomycetes					
Order- Hypocerales					
1	<i>Acremonium murorum</i> (Corda) W. Gams 1971	+	-	-	+
2	<i>Cosmospora butyri</i> (J.F.H. Beyma) Gräfenhan, Seifert & Schroers 2011	+	-	-	-
3	<i>Fusarium proliferatum</i> (Matsush.) Nirenberg ex Gerlach & Nirenberg 1982	+	-	-	-
4	<i>F. redolens</i> Wollenw. 1913	+	+	-	-
5	<i>Gliomastix cerealis</i> (P. Karst.) C.H. Dickinson 1968	+	+	+	+
6	<i>Rectifusarium ventricosum</i> (Appel&Wollenw.) L. Lombard &Crous 2015	-	+	+	-
Order- Sordariales					
1	<i>Humicola fuscoatra</i> Traaen 1914	-	+	+	-
2	<i>Trichocladium griseum</i> (Traaen) X. Wei Wang & Houbraken 2018	-	+	+	+

Table 1 Continued.

Sl. No.	Endophytic fungal isolates	L	S	R	PC
Order- Incertaesedis					
1	<i>Arthrinium arundinis</i> (Corda) Dyko & B. Sutton 1979	+	-	-	+
Order- Glomerellales					
1	<i>Colletotrichum gloeosporioides</i> (Penz.) Penz. & Sacc. 1884	+	-	-	+
Class- Dothiideomycetes					
Order- Pleosporales					
1	<i>Alternaria alternata</i> (Fr.) Keissl. 1912	-	+	+	-
2	<i>Juxtiphoma eupyrena</i> (Sacc.) Valenz.-Lopez, Crous, Stchigel, Guarro & Cano 2017	+	+	+	+
Order- Capnodiales					
1	<i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries 1952	+	+	+	+
Order- Tubeufiales					
1	<i>Tubeufia cerea</i> (Berk. & M.A. Curtis) Höhn. 1919	+	-	+	-
Class- Eurotiomycetes					
Order- Eurotiales					
1	<i>Penicillium brevicompactum</i> Dierckx 1901	+	+	+	+
2	<i>P. glabrum</i> (Wehmer) Westling 1911	+	-	+	+
3	<i>P. simplicissimum</i> (Oudem.) Thom 1930	+	+	+	-
4	<i>P. jensenii</i> K.W. Zaleski 1927	-	-	-	+
5	<i>Talaromyces islandicus</i> (Sopp) Samson, N. Yilmaz, Frisvad & Seifert 2011	-	-	-	+
6	<i>T. purpureogenus</i> Samson, N. Yilmaz, Houbraken, Spierenb., Seifert, Peterson, Varga & Frisvad 2011	+	+	+	+
7	<i>T. ruber</i> (Stoll) N. Yilmaz, Houbraken, Frisvad & Samson 2012	+	+	+	+
8	<i>T. rugulosus</i> (Thom) Samson, N. Yilmaz, Frisvad & Seifert 2011	+	+	+	+
Class- Pezizomycetes					
Order- Pezizales					
1	<i>Phymatotrichopsis omnivore</i> (Duggar) Hennebert 1973	-	+	-	-
Class- Leotiomycetes					
Order- Incertaesedis					
1	<i>Scytalidium lignicola</i> Pesante 1957	-	-	+	-
Phylum: Oomycota- 3 Genera, 5 Species					
Class- Incertaesedis					
Order- Peronosporales					
1	<i>Globisporangium intermedium</i> (de Bary) Uzuhashi, Tojo & Kakish. 2010	-	-	+	-
2	<i>G. irregular</i> (Buisman) Uzuhashi, Tojo & Kakish. 2010	+	+	+	+
3	<i>Phytophthora cactorum</i> (Lebert & Cohn) J. Schröt. 1889	-	-	-	+
4	<i>P. cinnamomi</i> Rands 1922	+	+	-	+
5	<i>Pythium aphanidermatum</i> (Edson) Fitzp. 1923	-	-	+	-
Phylum: Zygomycota- 3 Genera, 5 Species					
Class- Mortierellomycetes					
Order- Mortierellales					
1	<i>Mortierella</i> sp. 1	+	-	-	-
2	<i>Mortierella</i> sp. 2	-	-	-	+
Class- Mucoromycetes					
Class- Mucorales					
1	<i>Gongronella butleri</i> (Lendn.) Peyronel & Dal Vesco 1955	+	-	-	-
2	<i>Rhizopus microspores</i> Tiegh. 1875	-	-	+	-
3	<i>Rhizopus</i> sp.	-	+	-	-
Mycelia sterilia (MS)- 5 isolates					
1	MS (Black)	-	+	-	-
2	MS (Brown)	-	+	+	+
3	MS (Red)	-	+	-	-
4	MS (White)	+	+	+	+
5	MS (Yellow)	+	-	+	-
Total- 23 Genera, 34 Species and 5 Mycelia sterilia					

L = leaf, S = stem, R = root, PC = pitcher cup, '+' = present and '-' = absent

Table 2 Diversity indices of fungal endophytes isolated from *N. khasiana* during the study period of 2015-2016.

Plant tissue	Shannon index (H')	Simpson's index of Dominance (D)	Simpson,s diversity index (1-D)	Species richness (S/√N)	Species evenness (E)
Leaf	2.78	0.07	0.92	1.64	0.96
Stem	2.68	0.08	0.91	2.07	0.92
Root	2.68	0.09	0.90	2.26	0.89
Pitcher cup	2.76	0.07	0.90	2.34	0.90

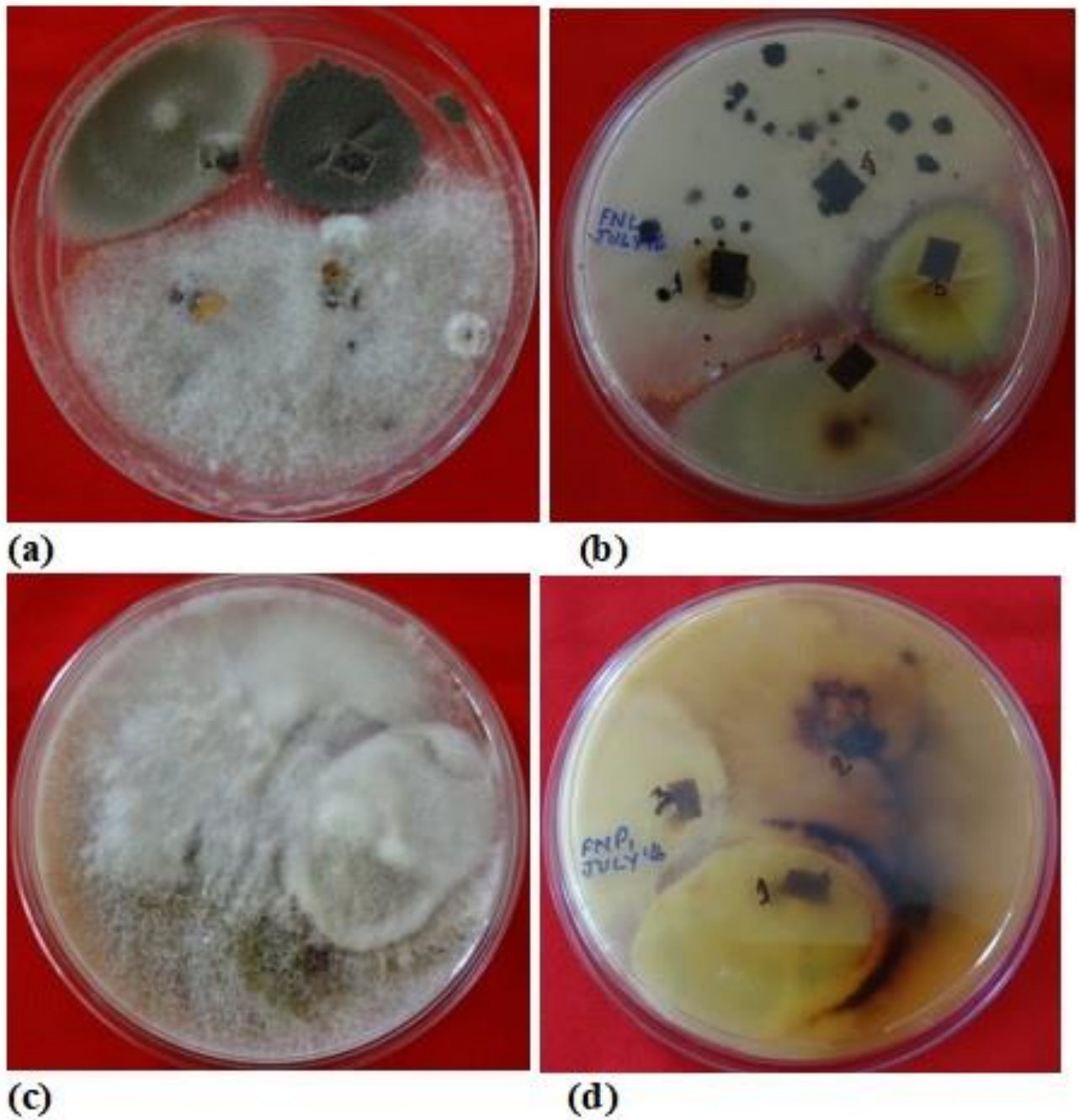


Fig. 1 – Plates showing mix cultures of fungal endophytes isolated from different plant parts of *N. khasiana*.

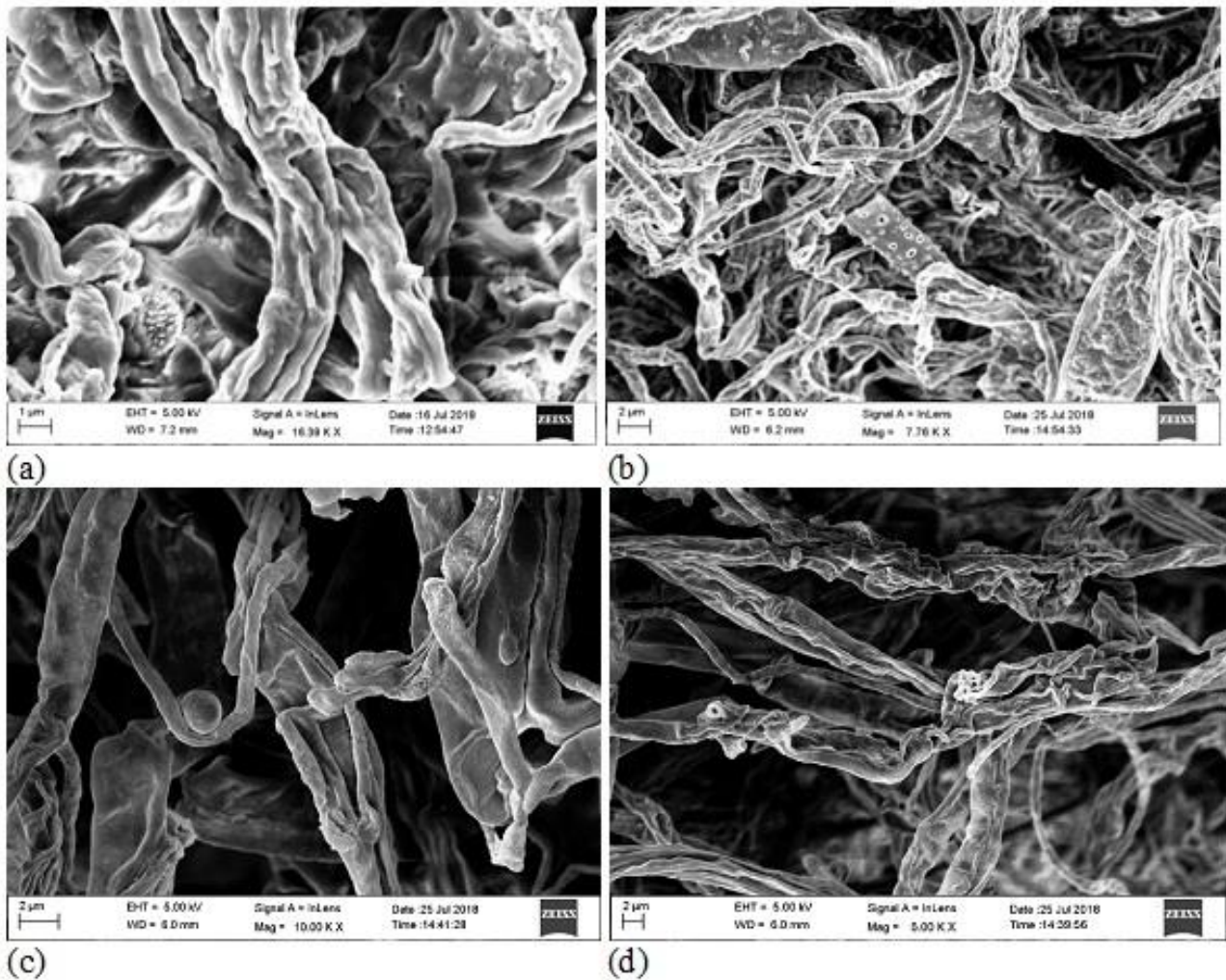


Fig. 2 – SEM micrograph showing the presence of endophytic fungal spore in (a) leaf and (b) stem; the elongation of new hyphae from mycelia of fungal endophytes in (c) root and (d) pitcher cup.

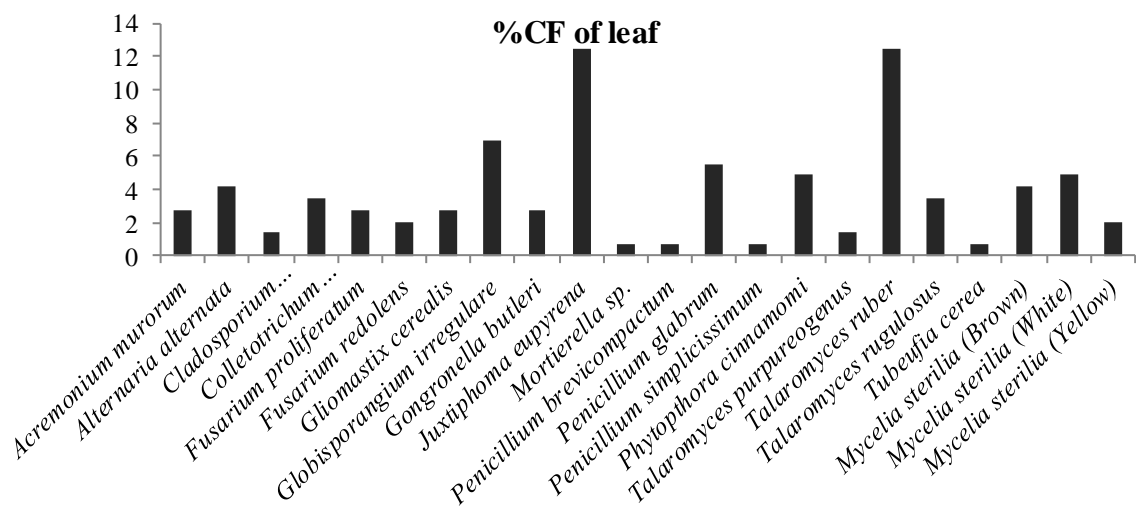


Fig. 3 – Percentage colonization frequency of endophytic fungi isolated from the leaves of *N. khasiana* during the study period of 2015-2016

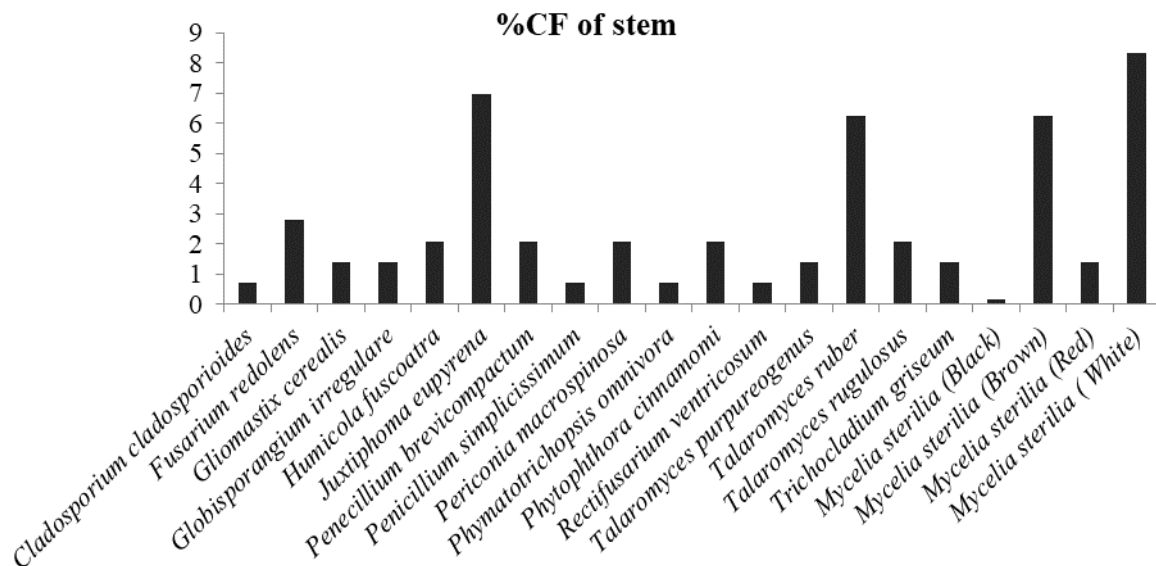


Fig. 4 – Percentage colonization frequency of endophytic fungi isolated from the stem of *N. khasiana* during the study period of 2015-2016

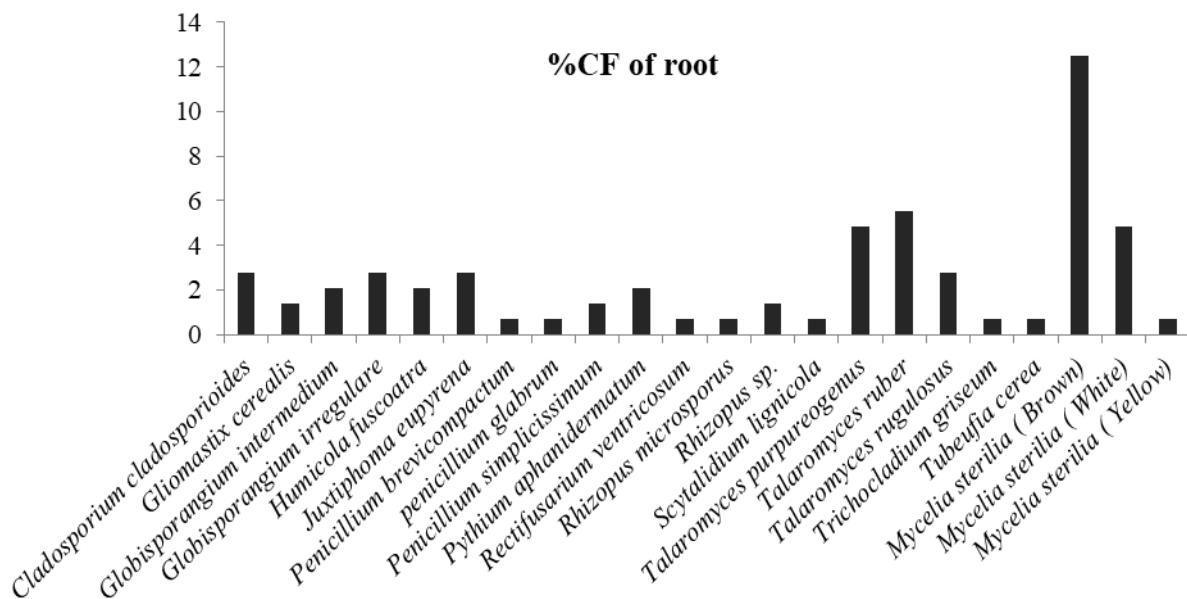


Fig. 5 – Percentage colonization frequency of endophytic fungi isolated from the root of *N. khasiana* during the study period of 2015-2016

Conclusion

From the present investigation, it can be concluded that the carnivorous plant *Nepenthes khasiana*, which is one of the endemic plants of the north-eastern state of Meghalaya, India, harbor a great diversity of endophytic fungi. The colonization frequency of the isolates from different tissues of the host plant significantly differed between leaf, stem, root and pitcher cup. Hence, this entire study can be considered as an earnest attempt in exploring the diversity of fungal endophyte and their association with the pitcher plant, and therefore, the further study to explore the facts that how these fungal endophytes are helping the plant in the digestion of prey, synthesis of several secondary metabolites, endurance towards the harsh environment, etc. is

much needed. Additionally, isolated endophytic fungal strains may also be evaluated for their antagonistic activity against plant pathogen of the host plant.

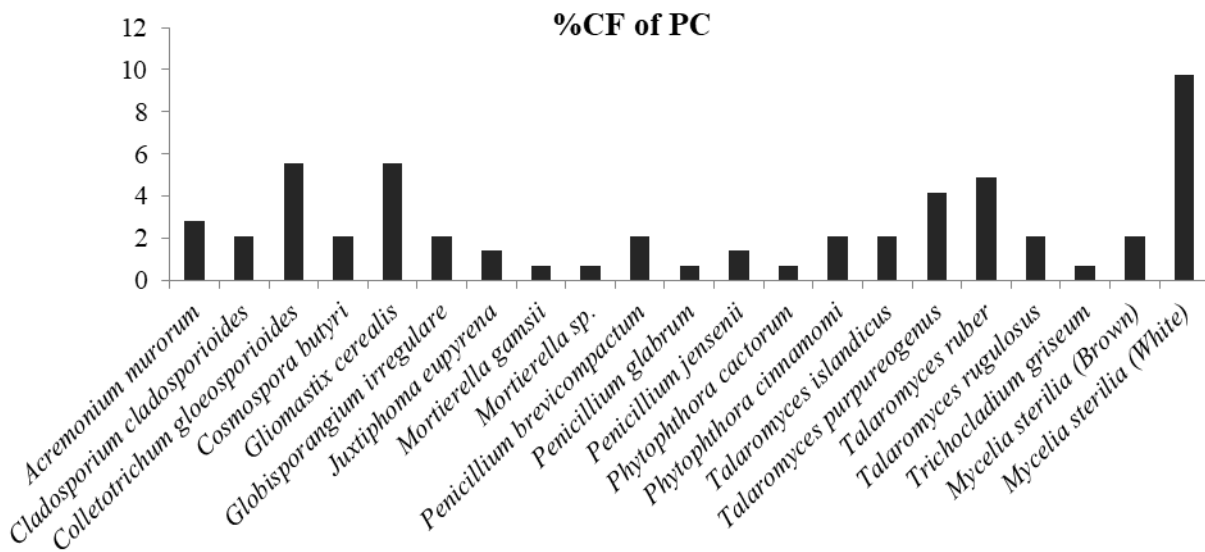


Fig. 6 – Percentage colonization frequency of endophytic fungi isolated from the pitcher cup (PC) of *N. khasiana* during the study period of 2015-2016.

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Conflict of interest

The authors declare no conflict of interest.

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