SEASONAL VARIATION AND DIVERSITY OF ENDOPHYTIC FUNGI FROM DIFFERENT PARTS OF *CENTELLA ASIATICA* (L.) URBAN IN DHAKA CITY, BANGLADESH

Bioresearch Communications
Volume 9, Issue 2, July 2023

DOI:
doi.org/10.3329/brc.v9i2.67077

Fazilatun Nessa[1], Sarowar Hosen[2] and Shamim Shamsi*

[1,2,*]Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh.

ABSTRACT

A total of 16 species of endophytic fungi were isolated from *Centella asiatica* plant. The fungi were *Aspergillus flavus, A. fumigatus, A. niger, A. terreus, Cladosporium* sp., *Colletotrichum* sp., *Curvularia hominis, C. lunata, Curvularia* sp., *Fusarium falsiforme, F. phaseoli, Monodictys putridinis, Penicillium commune, Penicillium* sp.1, *Penicillium* sp.2, *Talaromyces trachyspermus.* Among them all the fungal species were found in summer season and 12 species in winter season. The highest value of Shannon and Simpson diversity index was found in the root in summer season and the lowest value of Shannon and Simpson diversity index was found in the stem in winter season. Maximum species richness of endophytic fungi was recorded in leaf in Margalef's and Menhinick index in winter season. Minimum species richness of endophytic fungi was recorded in stem in Margalef's and Menhinick index during summer season.

KEYWORDS: Endophytic fungi, *Centella asiatica*, Species richness, Species diversity.

RECEIVED: 09 February 2023, ACCEPTED: 14 May 2023

TYPE: Original Research

CORRESPONDING AUTHOR: Dr. Shamim Shamsi
Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh.
Email: botanyshamsi@du.ac.bd

Introduction

Endophytes are microorganisms that colonize living internal tissues of plants without causing any immediate overt symptoms (Petrini 1986). These endophytic fungi reside within the living tissues of the host and establish relationships with the host ranging from symbiotic to pathogenic. Each plant species hosts one or more endophytic fungal species. Endophytic fungi are diverse polyphyletic groups of microorganisms and can thrive asymptomatically in different healthy tissues of living plants above and under the ground parts, including stems, leaves and roots (Faeth and Fagan 2002). Endophytic fungi are a highly biodiverse and versatile microbial community that seem to be ubiquitous in nature. Studies have shown that almost all plants contain endophytic fungi (Ding *et al.* 2015; Jin *et al.* 2021). They have been isolated and cultured from the roots and above-ground parts of various plants.

Centella asiatica is commonly known as "Thankuni" (in Bangladesh) or Indian pennywort-that is a tropical plant native to Southeast Asian countries. It is an herbaceous plant belonging to the family Apiaceae. It has a long history of use in the traditional Ayurvedic medicine systems in Bangladesh and India. Medicinal plants growing in natural habitats are promising hosts of endophytic fungi. Different plant parts, especially the leaves, stems and roots are considered an enormous repository of these fungal endophytes with reported cytotoxic, antifungal, antiviral, and antimicrobial activities (Strobel *et al.* 2003; Tolulope *et al.* 2015; Ibrahim *et al.* 2016). The study will shed the light on the diversity of endophytic fungi of *Centella asiatica* in relation to plant parts and to evaluate the seasonal variation of endophytic fungal populations in relation to their abundance.

Materials and Methods

Plant Materials

Mature and healthy leaf, stem and root samples of *Centella asiatica* (L.) Urban were collected from the botanical garden of Curzon Hall Campus, University of Dhaka. Samples were collected in different seasons (summer and winter).

Isolation of endophytic fungi

Fungal isolation was done using the "Tissue planting method" (CAB 1968) on PDA (Potato Dextrose Agar) medium. The samples were treated within 48 hours after collection. The preserved leaf, stem and root samples of *C. asiatica* were rinsed in running tap water, cut into small segments about 3×3 mm^2 in size with the help of a sterilized scissor/blade under aseptic conditions and was immersed in a 2 - 4% aqueous solution of sodium hypochlorite (NaOCl, Clorox solution) for 1 to 1.5 minutes. Then the samples were rinsed out three times with sterile distilled water and allowed to surface dry on sterilized filter papers inside Petri plates under aseptic

conditions. The surface sterilized plant segments were then placed on sterilized Petri plates containing potato dextrose agar medium (PDA). Petri plates containing PDA medium with inocula were incubated at 25 ± 2ºC temperature in the incubation chamber. Any hyphae that extended from the leaf fragments were used in a successive culture with PDA.

Identification of fungi

Morphological studies of the isolated fungi were done to identify the fungi primarily depending on the colony colour, texture, shape, diameter, surface appearance following standard literatures (Benoit *et al*. 1970; Booth 1971; Ellis 1971, 1976; Barnett and Hunter 1972).

Molecular identification of the isolates was performed using the internal transcribed spacer (ITS) region. PCR amplification was conducted using the ITS1 as forward and ITS4 as reverse primers. The PCR was initiated by an initial denaturation step at 94°C for 5 minutes following 30 cycles of 94°, 54° and 72°C each for 30 sec, with a final extension step of 5 min at 72°C and ended with 4°C. Sequence alignment and editing were done with the BioEdit Sequence Alignment program and compared against the sequences already available in the databases using the programme BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

Data Analysis

Determination of percent frequency of the isolated endophytic fungi

The percent frequency of the fungi was calculated following the standard formula given below:

$$Percent\ frequency = \frac{Total\ number\ of\ colonies\ of\ the\ same\ fungi}{Total\ number\ colonies\ of\ different\ fungi} \times 100\%$$

Analysis of Diversity Indices

Three indices were used to estimate species diversity, species richness and species evenness following Ludwick and Reynold (1998).

Result and Discussion

In Tissue planting method, a total of 16 species of endophytic fungi were isolated from *C. asiatica* plant (**Fig. 1**). The fungi were *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Cladosporium* sp., *Colletotrichum* sp., *Curvularia hominis*, *C. lunata*, *Curvularia* sp., *Fusarium falsiforme*, *F. phaseoli*, *Monodictys putridinis*, *Penicillium commune*, *Penicillium* sp.1, *Penicillium* sp.2, *Talaromyces trachyspermus* (**Fig. 2, Table 1**)

Figure 1. a. *Centella asiatica* in nature, b. different parts of *C. asiatica*

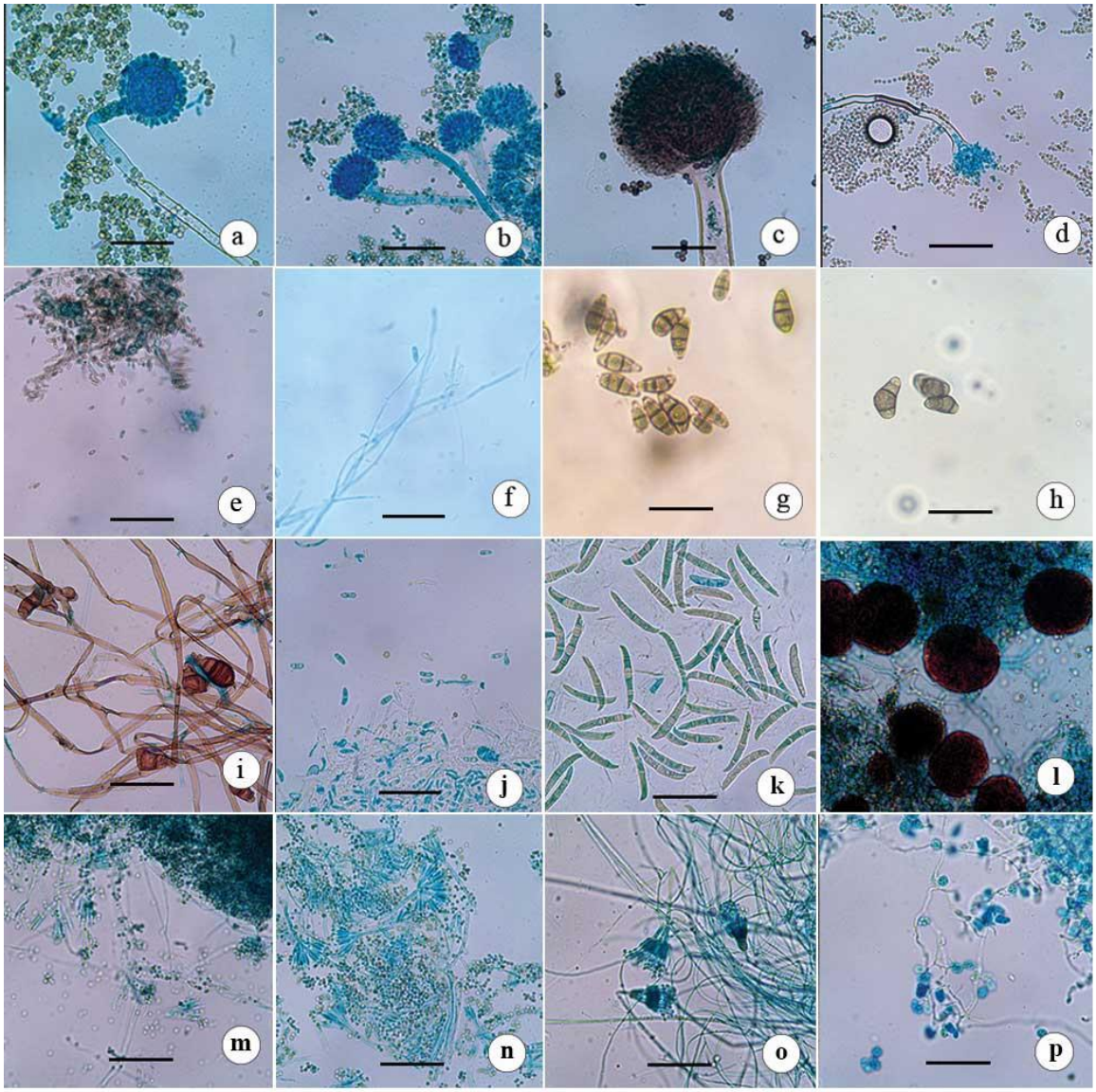

Figure 2. Conidia under microscope a. *Aspergillus flavus,* b. *A. fumigatus,* c. *A. niger,* d. *A. terreus,* e. *Cladosporium* sp., f. *Colletotrichum* sp., g. *Curvularia hominis,* h. *C. lunata,* I. *Curvularia* sp., j. *Fusarium falsiforme,* k. *F. phaseoli,* l. *Monodictys putridinis,* m. *Penicillium commune,* n. *Penicillium* sp.1, o. *Penicillium* sp.2, p. *Talaromyces trachyspermus.* (Bar = 50 µm)

Table 1. Endophytic fungi associated with leaf, stem and root of *Centella asiatica.*

SerialNo.	Nameof fungi	Leaf	Stem	root
1.	*Aspergillus niger*	+	+	+
2.	*A. flavus*	+	+	+
3.	*A. fumigatus*	+	-	+
4.	*A. terreus*	+	+	-
5.	*Cladosporium* sp.	+	+	+
6.	*Colletotrichum* sp.	+	-	-
7.	*Curvularia* sp.	-	+	-
8.	*C. hominis*	+	-	+
9.	*C. lunata*	-	+	-

10.	*Fusarium falsiforme*	+	-	+
11.	*F. phaseoli*	-	+	+
12.	*Monodictys putridinis*	-	-	+
13.	*Talaromyces trachyspermus*	-	-	+
14.	*Penicillium commune*	-	+	-
15.	*Penicillium* sp.1	+	+	+
16.	*Penicillium* sp.2	+	-	+

'+' and '–' represent the presence and absence of fungi respectively.

In *C. asiatica* leaf, the highest frequency was found in *A. niger* (21.54%) and the lowest frequency in *Curvularia hominis* (3.37%) during summer season; the highest frequency was found in *Penicillium* sp.1 (30.80%) and the lowest frequency in *A. terreus* (6.06%) in winter season. *Penicillium* sp.2 was found only in winter season and *Curvularia hominis* was found only in the summer season.

In stem, the highest frequency was found in *Cladosporium* sp. (24.25%) and the lowest frequency in *Curvularia lunata* (1.85%) during summer season; in winter season, the highest frequency was found in *Penicillium* sp.1 (35.02%) and the lowest frequency in *A. niger* (8.59%). *A. terreus, Curvularia lunata, Fusarium phaseoli* were found only in summer season.

In root, the highest frequency was found in *Cladosporium* sp. (21.91%) and the lowest frequency in *Talaromyces trachyspermus* (1.75%) during summer season; in winter season, the highest frequency was found in *Penicillium* sp.1 (36.75%) and the lowest frequency in *Curvularia hominis* (4.60%). *Monodictys putridinis* and *Talaromyces trachyspermus* were only found during summer season. (Table 2)

Table 2. Percent frequency of endophytic fungi associated with the leaf, stem and root of *Centella asiatica* at different seasons.

Name of fungi	**Percent frequency of fungi**					
	leaf		Stem		Root	
	Summer	Winter	Summer	Winter	Summer	Winter
Aspergillus niger	21.54	15.17	12.33	8.59	13.97	9.76
A. flavus	6.95	10.51	13.66	9.76	5.09	7.94
A. fumigatus	15.65	2.56	-	-	12.21	7.22
A. terreus	4.99	6.06	6.30	-	-	-
***Cladosporium* sp.**	14.13	7.35	24.25	18.10	21.19	6.98
***Colletotrichum* sp.**	10.32	9.70	-	-	-	-
Curvularia hominis	3.37	-	-	-	12.21	4.60
C. lunata	-	-	1.85	-	-	-
***Curvularia* sp.**	-	-	10.11	13.22	-	-
Fusarium falsiforme	10.55	7.47	-	-	13.97	9.60
F. phaseoli	-	-	17.84	-	-	-
Monodictys putridinis	-	-	-	-	7.21	-
Talaromyces trachyspermus	-	-	-	-	1.75	-

Penicillium commune	-	-	7.89	15.32	-	-
***Penicillium* sp. 1**	12.51	30.80	5.77	35.02	7.04	36.75
***Penicillium* sp. 2**	-	10.38	-	-	5.36	17.14

'–' represents no mycelial growth of respective fungi.

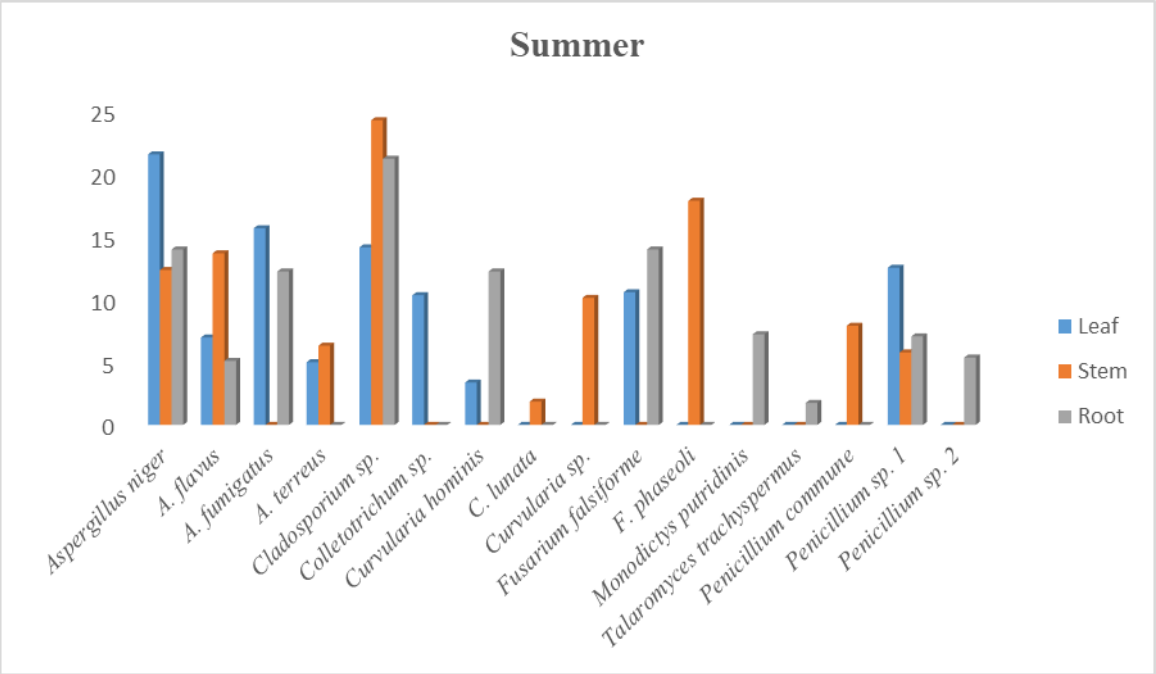

Figure 3. Percent frequency of endophytic fungi associated with the leaf, stem and root of *Centella asiatica* during summer.

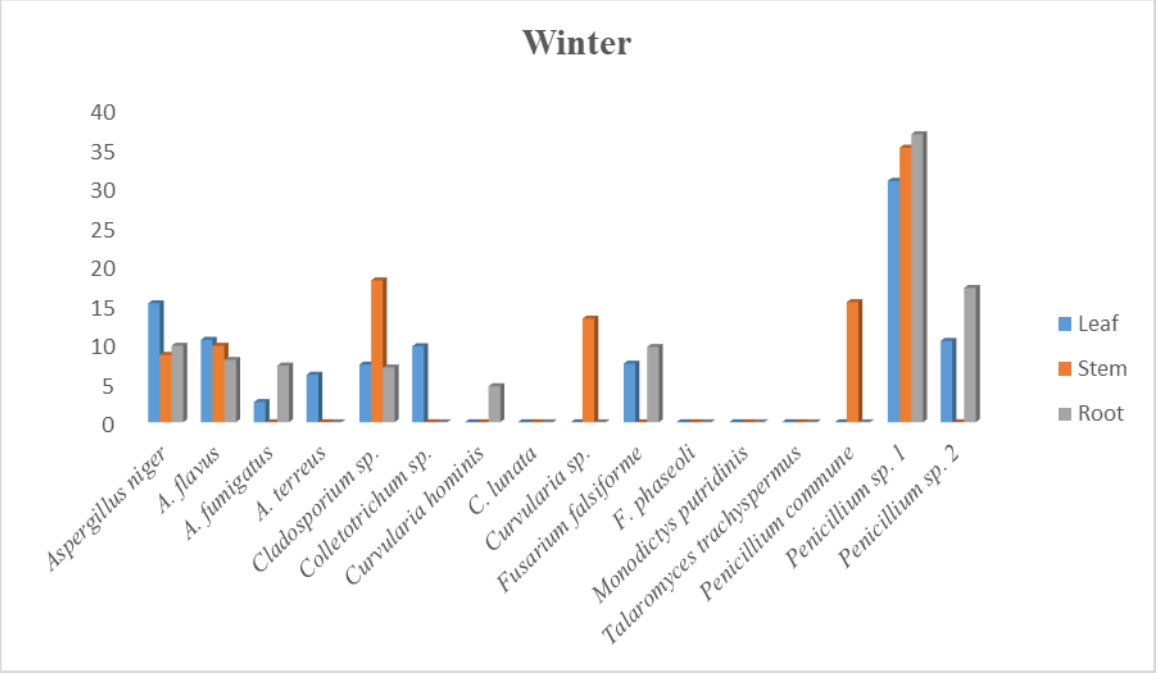

Figure 4. Percent frequency of endophytic fungi associated with the leaf, stem and root of *Centella asiatica* during winter.

The highest value of Shannon diversity index (2.15) and Simpson diversity index (0.89) was found in the root in summer season, which indicates high endophytic fungal species diversity in present study and the lowest value of Shannon diversity index (1.67) and Simpson diversity index (0.81) was found in the stem in winter season which indicates low endophytic fungal species diversity in present study. The highest value of Shannon evenness index was found in the leaf during summer season (0.949) which indicates the number of endophytic fungal species within this plant parts was fairly constant and the lowest value was found in the root in winter season (0.887) which indicates the number of endophytic fungal species within this plant parts was not constant. Maximum species richness of endophytic fungi was recorded in leaf with 2.456 in Margalef's index and 1.6013 in Menhinick index during winter season indicating the increase of endophytic fungal biodiversity. Minimum species richness of endophytic fungi was recorded in stem with 2.045 in Margalef's index and 1.2728 in Menhinick index during summer season indicating the decrease of endophytic fungal biodiversity. **(Table 3)**

Table 3.Shannon diversity index (H) and Simpson diversity index (D) of endophytic fungi.

Parts of plant	Season	Shannon diversity index (H)	Simpson diversity index (D)	Shannon Evenness index (E)	Margalef index (R1)	Menhinick index (R2)
Leaf	Summer	2.09	0.88	0.949	2.226	1.3245
	Winter	1.99	0.86	0.907	2.456	1.6013
Stem	Summer	2.03	0.87	0.923	2.045	1.2728
	Winter	1.67	0.81	0.935	2.308	1.591
Root	Summer	2.15	0.89	0.933	2.226	1.3245
	Winter	1.85	0.84	0.887	2.423	1.5617

This study indicates that the diversity of the endophytes can be different with the plant parts. Depending on sampling season, 16 species were identified in summer and 12 species in winter. More than 600,000 species of endophytic fungi are theorized to exist worldwide (Schmit *et al.* 2007) and various scientific approaches have been used to detect endophytic fungi to date. In this study, Shannon diversity index (H) was the highest in root, with the greatest number of endophytes being isolated during the summer season. Hence, climate probably affects endophytic dispersal (Schulz *et al.* 2005). These results also indicate that the number of endophytic fungal morphotypes differ across the season.

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