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A relative study of diversity of endophytic fungi in a Lianas *Butea superba* from Belpahari and their seasonal variation

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Abstract

Fungal endophytes from *Butea superba* were studied collecting from Belpahari of Jhargram district of West Bengal during three seasons-winter, summer and monsoon. A total of 159 plant tissue segments were resided by endophytic fungi among 225 tissue segments and endophytic fungi of the number of 201 were isolated from 159 various tisue specimens such as leaf, petiole and stem. The fungal isolates belong to the genera as many as 25, along with 5 unidentified fungi and few sterile mycelia. *Beltrania* sp., *Lasiodiplodia* sp., *Fusarium* sp., *Penicillium* sp., *Pestalotiopsis* sp. were found most commonly and dominantly found out of all isolates. Majority of the endophytic isolates were during winter season and least in summer. Colonization frequencies in winter, summer and monsoon are 73.33%, 60% and 78.66% respectively. This difference in CF % might be due to variation of moisture content in various seasons inside it and variation of absorbable nutrient contents in forest floor. It has been observed that microenvironment and microclimate were determining factors for assuring endophytic biodiversity.

Keywords: Endophytes, diversity, fungi, indices, lianas.

Introduction

Anton De Bary, 1866 was the first man who used and introduced the term `endophyte' and was applied to 'the organisms that occur within plant tissues'. Endophytic fungi are microorganisms that live within the inner tissue of plants without causing apparent symptoms (Wilson, 1995). Although endophytic fungi are primarily mutualistic and commensalistic symbionts, they may not continue as endophytes throughout their life cycles (Porras-Alfaro and Bayman, 2011). Endophytes are ubiquitous in idistribution. Endophytic fungi that infest plants were found in all environments studied (Caroll, 1988; Petrini, 1991). Microorganisms that colonize internal plant tissues without causing any diseases symptoms or apparent injury are called endophytes (Bacon et al., 2000). Many fungal, bacterial, actino-mycetean members are endophytes but most frequently isolated endophytes are fungi (Strobel et al., 2003). Carrol and Carrol, (1988) reported that endophytes live without any symptoms and sometimes systematically within the plant tissues. They have been found infested with every plant species

investigated so far. It is believed that plants from unique environmental settings and which are endemic are likely to accommodate distinct endophytic microorganisms as well as microorganisms making novel bioactive products (Daisy et al., 2003). Others are present in the intercellular-space of leaves, petioles and inner tissues of stems (Van Wyk et al., 1990; Verstraete et al., 2011). Lianas plants are woody climbers which grow supporting another straight and strong long trees and cover the topmost canopy of it. Different lianas plants harbour some distinct fungal endophytes that are believed to be associated with the production of antimicrobial substances (Banerjee et al., 2006). Fungal endophytes in *Theobroma cacao* and *Solanum melongena* reduced foliar and root diseases respectively, and treatment of *Glycine max* with culture filtrate of endophyte- *Cladosporium sphaerospermum* increased plant height (Mejia et al., 2008; Narisawa et al., 2002; Hamayun et al., 2009). Duong et al., (1994) studied fungal endophytic diversity and community patterns in healthy and yellowing leaves of *Citrus lemon*. Gilbert et al., (2007) reported fungal symbionts in tropical trees.

Despite the largest diversity of endophytic species in tropical and subtropical rainforests, their biodiversity in tropical country is still poorly studied. Some researchers isolated very diverse groups of endophytic fungi from plant tissues (Arnold et al., 2001).

Endophytes are the normal microflora of the plant tissues (Ganley et al., 2004). They protect the plants against pests. They also enhance the defense mechanisms of host plant against unfavourable environments. Endophytic fungi show considerable antibacterial and antifungal activity (Jena et al., 2013). Various antifungal agents have been explored, but the control of many of the fungal diseases has not been achieved.

The goal of the study was to identify the fungal endophytic communities in leaves, petioles and stems of *Butea superba*. The objectives are to: (i) isolate the endophytic fungi (ii) determine the diversity of endophytic fungi (iii) compare the endophytic fungal isolation during various seasons and compare the endophytic diversity pattern (iv) to determine host organ specificity of fungal endophytes.

Materials and methods

Study sites and collection of samples

The study was conducted in Jhargram district of West Bengal, one lianas plant- *Butea superba* (Papilionaceae) was selected from Belpahari forest area for endophytic fungal screening. The district is situated in between the latitude of 22°25′ to 22°57′North and longitude of 87°11′East. The altitude is 23M above from the sea level. The climate is tropical, warm and humid with a mean temperature of 33°C and an average rainfall of 120cm.

Sampling procedure

Plant samples (leaves, stems, petioles) were collected randomly from healthy, disease-free mature plants from during winter, summer and monsoon. The samples immediately after collection were kept in zipper-lock plastic packets, brought to the laboratory and were processed within a few hours or stored at 4^{o}C within 2-3 hours of collection until isolation procedure was accomplished.

Surface disinfection

Samples collected from different localities were thoroughly washed under running tap water before processing and following

sequences were followed: leaf, petiole and stem samples were surface sterilized by sequentially dipping into 70% ethanol for 1 min, 1% sodium hypochlorite (NaOcl)(4% available chlorine) for 4 min, 90% ethanol for 20sec. Finally, samples were rinsed with sterile distilled water for 3 times, then allowed to surface dry under sterile condition.

Placing the samples in media

Sterile leaves were cut into pieces of about 1 square cm size by sterile scissor and placed in plate of water agar (WA), 5 samples in each, equidistant from each other. Similarly 5 sterile petioles of 0.5-1cm long were placed in another WA plate. Stem tissues were cut into short pieces of 4-5 cm long and after sequential sterilization, the outer layer was removed and inner tissues were peeled with sterile scalpel. Thin peels from various depth were placed on another WA plate. Thus, at least 5 replica plates for each sample from the plant of one locality were made.

Isolation of endophytic fungi

After placing the samples fungal growth was observed each and every day. Within 2-3 days fungal hyphae were in appearance. Some samples show more than one hyphal growth. From each sample fungal hypha was isolated and transferred to PDA media by cutting a square block of water agar. The plates were incubated in light chamber at 23^{o}C. After 10-15 days huge mycelial and in some cases reproductive growth was observed. Culture slants were made and preserved for identification at 4^{o}C and also for further work in future.

Identification of endophytes

The endophytic fungal organisms were studied under optical compound microscope. The fungal isolates were identified based on their morphological and reproductive characters using the standard identification manuals (Barnett et al., 1998; Ellis Martin et al., 1997; Gilman, 2001; Magurran, 2004).

Data analysis

The relative colonization frequency (CF%) was calculated as the number of sample segments colonized by at least a fungus divided by total number of segments plated x100 using the formula outlined by Hata and Futai: CF = (N_{col}/N_t x100, where N_{col} = number of segments colonized by at least a fungus, N_t = total number of segments plated. Dominant endophytes were calculated as percentage of colony frequency divided by sum of percentage of colony frequency of all endophytes x100. Dominant endophyte percentage (D) = N_i/N_s x100, where N_i = percentage of colony frequency of individual endophytes, N_s = percentage of colony frequency of all endophytes. Using PAlaeontological STatistics software package (PAST), following diversity indices were calculated:

(a) Simpson's Diversity Index (1-Dominance) was calculated using the formula 1-D, where D = ∑n(n-1)/N(N-1). Here, n = the total number of organisms of a particular species, N = the total number of organisms of all species.

(b) Shannon-Wiener diversity index was calculated using the following formula: Shannon-Wiener index (H') = -∑s(Pi)(In Pi), where H' = Symbol for the diversity in a sample of species or kinds, s = the number of species in the sample, Pi = relative abundance of ith species or kinds and measured by = n/N, N = total number of individuals of all kinds, n_i = number of individuals of its species, In = log to the base 2.

(c) Evenness was calculated using the following formula: Evenness (E) = H'/H'max, where H'max is the maximum value of diversity for the number of species.

Result and discussion

In winter study of *Butea superba*, total 75 plant segments were plated out of which 53 were infested with endophytic fungi and 53 fungi were isolated from them. In summer study, total 75 plant segments were plated out of which 38 were infested with endophytic fungi and 38 fungi were isolated from them and in monsoon study out of 75 segments 72 were infected by fungi and 72 isolates were found. In monsoon colonization frequency was maximum (96%) and in summer was minimum (50.64%). Among various tissues, the maximum number of endophytes were found in petiole (90.66%) and minimum in leaf (46.66%). Suryanarayanan and Rajagopal, (2000) isolated 963 fungi from bark sample of 10 tropical tree species in southern India. Banerjee et al., (2009a), reported 14 endophytic fungal genera in 3 medicinal herbs. Only 6 different fungal genera and few unidentified genera with sterile mycelia were found. Among all isolated fungi during different seasons, *Fusarium* sp. was maximum in number (98). *Arthrinium* sp. and *Verticillium* sp. also found more in number. Shannon-Wiener index was maximum in summer (1.259) and Shimpson's diversity (0.5956) was also maximum. Collado et al., (1999) showed geographical and seasonal influences on the distribution of fungal endophytes.

To protect from the heat effect, fungi inhabit in a deep region of tissue than surface area to survive themselves. That is why probably stem tissue rather than leaf and petiole had maximum endophytic fungal colonization. Among the isolated endophytic fungi, the maximum number of *Fusarium sp*. were found in all types of tissue and was more or less equally distributed in all plant segments of all seasons. From the graph it was observed clearly that the highest diversity of isolated endophytic fungi was found in plants during monsoon.

Table 1. Comparison of isolated fungi and colonization frequency (CF%) of isolated endophytic fungi from *Butea superba* in three seasons.

Seasons	Fungi isolated	CF
Winter	53	70.66%
Summer	38	50.64%
Monsoon	72	96%
Total	164	72.43%

Table 2. Diversity indices and species richness of endophytic fungi in *Butea superba* from Belpahari (Bel) during winter, summer and monsoon.

Parameters	Winter	Sum-mer	Monso-on
Species richness	5	6	5
Individuals	53	38	72
Simpson diversity	0.4763	0.5956	0.5457
Shannon-Wiener index	0.9327	1.259	1.081
Evenness	0.5083	0.5869	0.5896
Fisher-alpha diversity	1.354	2.004	1.232

Conclusion

Host-organ and tissue specificity has been observed in colonization of endophytes. There were diverse groups of fungal endophytes found in selected lianas plants in the study. Majority have been identified, with some remaining as unknown genera. In respect of seasons, total isolated fungi in all selected plants of investigation during winter, summer and monsoon were 53, 38 and 72 respectively.

Table 3. Colonization frequency of endophytes in various organs of *Butea superba* in Belpahari during various seasons.

Seasons	Total segments plated	Segments infested with fungi	Fungi isolated from the segments	Colonization frequency (CF%)	CF% in leaf	CF% in petiole	CF% in stem
Winter	75	53	53	70.66%	16%	96%	100%
Summer	75	38	38	50.64%	28%	76%	48%
Monsoon	75	72	72	96%	96%	100%	92%
Total	225	163	163	72.44%	46.66%	90.66%	80%

Table 4. Endophytic fungi isolated from leaf (L), petiole (P) and stem (S) segments of *Butea superba* from Belpahari during different seasons.

Isolated Endophytic fungi	Total	Winter			Summer			Monsoon		
		L	P	S	L	P	S	L	P	S
Alternaria sp.	1	0	0	0	0	0	1	0	0	0
Arthrinium sp.	19	0	0	6	1	1	3	1	4	3
Chaetomium sp.	2	0	0	0	1	1	0	0	0	0
Colletotrichum sp.	1	0	0	0	0	0	0	1	0	0
Fusarium sp.	98	4	24	9	4	12	7	18	13	16
Lasiodiplodia sp.	4	0	0	0	0	3	1	0	0	0
Mycelia sterilia	14	0	0	1	1	2	0	6	4	0
Unidentified	7	0	1	0	0	0	0	0	2	4
Verticillium sp.	8	0	0	8	0	0	0	0	0	0
Total	163	4	25	24	7	19	12	26	23	23

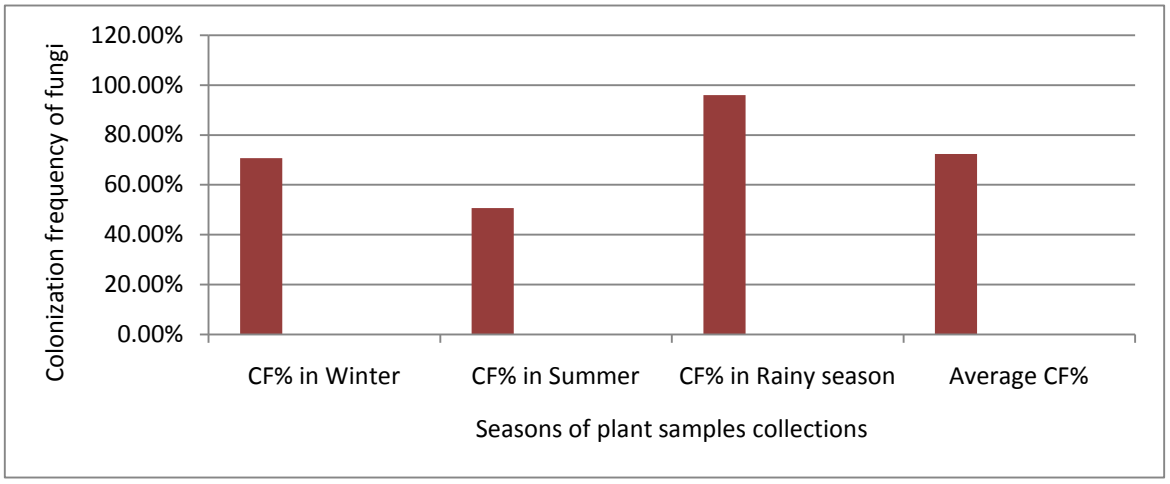

Figure 1. Comparison of colonization frequency (CF) of isolated endophytic fungi during various seasons.

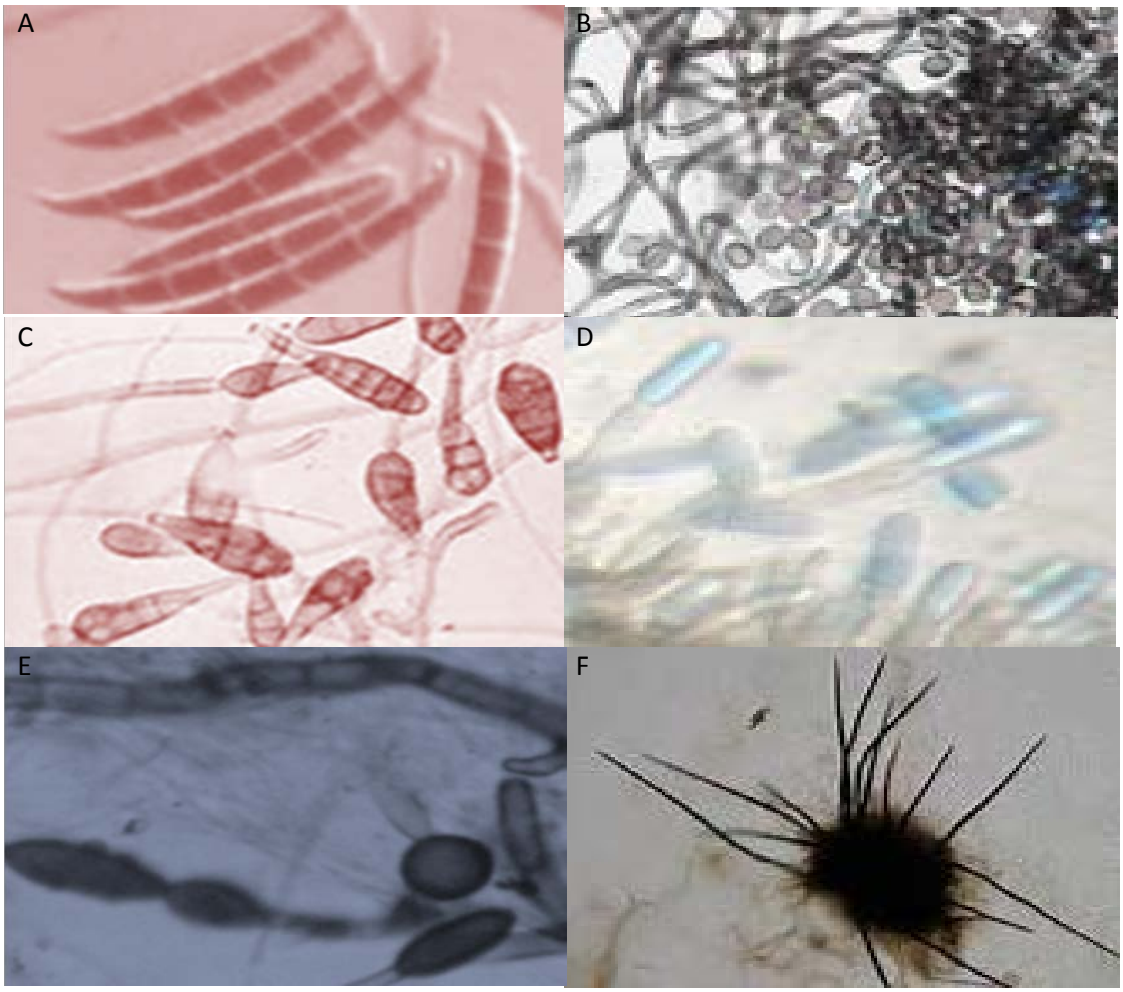

Figure 2. Microscopic view some isolated endophytes A. *Fusarium* sp. B. *Chaetomium* sp. C. *Alternaria* sp. D. *Lasiodiplodia* sp. E. *Arthrinium* sp. F. *Colletotrichum* sp.

The endophyte diversity in different types of tropical forests of southern India is considerably lower when compared to that of the neotropics, perhaps owing to low floristic diversity, presence of relatively open canopies, highly variable annual rainfall and dry-season ground fires (Murali et al., 2007). Species accumulation curves of foliar endophytes for these forest communities show that, while individual tree species have rich endophyte diversity, similar endophyte species are shared by different tree species (Suryanarayanan et al., 2003). It was found that the highest colonization of endophytes was in monsoon and lowest was in summer.

Conflict of interest

Authors declare that there is no conflict of interest.

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