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Diversity of Endophytic fungi in Lianas-butea Superba from West Medinipur District and its seasonal and regional variation

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ABSTRACT

To determine diversity of endophytic fungi associated with lianas from three different forest localities of West Medinipur district. Samples were collected randomly in various seasons. Fungi were isolated and identified based on mycelial shape and structure; sexual and asexual reproductive characters; attachment of spores and cultural conditions. A total of 428 endophytic isolates were obtained from 675 different sample segments. Colonization frequency is 63.39%. In Chilkigark highest number of endophytes have been isolated. In monsoon highest percentage (95.98%) were isolated. Dominant endophytic fungi are *Fusarium*, *Chaetomium*, *Lasiodiplodia*, *Verticillium*. Maximum endophytic isolates were obtained from stem segments followed by leaves and petioles. Among all endophytic fungi class Deuteromycetes were dominant over other fungal classes. Shannon-Weiner and Simpson's indices showed rich diversity of endophytic fungi. This indices suggest even and uniform occurrence of various species.

Key words : *Endophytes, Lianas, Diversity, Butea, Sacred grooves.*

Introduction

The term 'endophyte' was first introduced by Anton De Bary in 1866, to describe organisms that live inside the plant. The term includes all organisms that live symptomlessly within plant tissues at least for some period of their life cycle. Much has been written on endophytes; they have been defined in many ways and there have been many reviews and books on the subject. So what is the best definition for endophytes? The most commonly used and accepted definition is that of Petrini (1991), "All organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to the host", however, there are many alternatives - The term 'endophyte' was first introduced by Anton De Bary and was for some time applied to "any organisms occurring within plant tissues" De Bary (1866). "Endophytes are any fungi isolated from internal symptomless plant tissues" Cabral *et al.* (1993). "Fungi that form unapparent infections within leaves and stems of healthy plants" Carroll (1988). Although endophytic fungi are primarily mutualistic and commensalistic symbionts, they may not continue as endophytes throughout their life cycles Bayman (2011). Fungal species lives as symbionts within the tissues of plants Raviraja (2005). Others are present in the intercellular-space of leaves, petioles and inner tissues of stems Verstraete *et al.* (2011). They play a vital role in phosphate uptake in plant nutrition. It is well known that endophytic diversity is shaped by the host identity Johnston *et al.* (2012) and also known that the endophytic community depends on geo-

graphic situation Thomas *et al.* (2008) and seasonal variation. Only relatively few herbaceous plants and shrubs have as yet been examined till now. For the present study of endophytes and study of its seasonal and regional variation an woody lianas plant *Butea superba* has been selected from West Medinipur district of West Bengal.

Materials and Methods

Study sites and collection of samples-The study was conducted in West Medinipur district of West Bengal, India. The district is situated in between the latitude of 22°252 to 22°572 North and longitude of 87°112 East. The altitude is 23 M above from the sea level. The climate is tropical, warm and humid with a mean temperature of 33 °C and an average rainfall of 120 cm. Lianas plant *Butea superba,* Family-Fabaceae, was selected from three different forest areas for present study.

Sampling procedure- Plant samples (leaves, stems, petioles) were collected randomly from mature, healthy, disease-free plants from each location during winter, summer and monsoon. The samples immediately after collection were kept in zipper-lock plastic bags, brought to the laboratory and stored at 4 °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 80% ethanol for 1 min, 1% sodium hypochlorite (NaOcl) (4% available chlorine) for 4 min, 90% ethanol for 20 sec. Finally, samples were rinsed with sterile distilled water for 3 times, and 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). 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.

Isolation of endophytic fungi- After placing the samples, within 2-3 days fungal hyphae were in appearance. From each sample fungal hyphae were isolated and transferred to PDA media by cutting a square block of water agar. The plates were incubated in light chamber at 23 °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 °C and also for further work in future.

Identification of endophytes-The endophytic fungal organisms was 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.* (1996); Gilman (1971).

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. 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 calcu-

Table 1. Colonization frequency of endophytes in various organs of *Butea superba* in winter at three places

Place	Total segments plated	Segments infested with fungi	Fungi isolated from the segments	Colonization frequency (CF%)	CF% in leaf	CF% in petiole	CF% in stem
Belpahari	75	53	53	70.66%	16%	96%	100%
Chilkigarh	75	40	40	53.33%	32%	52%	76%
Godapiasal	75	28	28	37.33%	8%	40%	64%
Total	225	121	121	53.77%	18.66%	62.66%	80%

lated using the following formula: Shannon-Wiener index (H2) = -"s(Pi)(In Pi), where H2 = 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 ith species, In = log to the base 2.

(c) Evenness was calculated using the following formula: Evenness (E) = H2/H2max, where H2max is the maximum value of diversity for the number of species.

Results and Discussion

In winter study of *Butea superba*, Total 225 plant segments were plated out of which 121 were infested with endophytic fungi and 121 fungi were isolated from them. In Belpahari colonization frequency was maximum (70.66%) and in Godapiasal was minimum (37.33%). Among various tissues, the maxi-

Table 2. Endophytic fungi isolated from leaf (L), petiole (P) and stem (S) segments of *Butea superba* from different localities during winter.

Isolated Endophytic fungi	Total	Belpahari			Chilkigarh			Godapiasal		
		L	P	S	L	P	S	L	P	S
Arthrinium sp.	07	0	0	6	1	0	0	0	0	0
Chaetomium sp.	02	0	0	0	0	0	2	0	0	0
Fusarium sp.	81	4	24	0	1	13	9	1	7	13
Lasiodiplodia sp.	14	0	0	0	5	0	7	0	1	1
Mucor sp.	01	0	0	0	1	0	0	0	0	0
Mycelia sterilia	01	0	0	1	0	0	0	0	0	0
Unidentified	01	0	1	0	0	0	0	0	0	0
Verticillium sp.	14	0	0	8	0	0	1	1	2	2
Total	121	4	25	24	8	13	19	2	10	16

Table 3. Colonization frequency of endophytes in various organs of *Butea superba* in summer at three places

Place	Total segments plated	Segments infested with fungi	Fungi isolated from the segments	Colonization frequency (CF%)	CF% in leaf	CF% in petiole	CF% in stem
Belpahari	75	38	38	50.64%	28%	76%	48%
Chilkigarh	75	32	32	42.64%	16%	16%	96%
Godapiasal	75	21	21	28%	28%	8%	48%
Total	225	91	91	40.44%	24%	33.33%	64%

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

Isolated Endophytic fungi	Total	Belpahari			Chilkigarh			Godapiasal		
		L	P	S	L	P	S	L	P	S
Alternaria sp.	01	0	0	1	0	0	0	0	0	0
Arthrinium sp.	11	1	1	3	0	0	3	0	1	2
Chaetomium sp.	08	1	1	0	0	0	3	2	0	1
Cochliobolus sp.	01	0	0	0	0	0	1	0	0	0
Fusarium sp.	52	4	12	7	2	3	13	4	1	6
Lasiodiplodia sp.	07	0	3	1	1	0	1	0	0	1
Mycelia sterilia	05	1	2	0	0	1	1	0	0	0
Penicillium sp.	02	0	0	0	0	0	0	0	0	2
Verticillium sp.	04	0	0	0	1	0	2	1	0	0
Total	91	7	19	12	4	4	24	7	2	12

Table 5. Colonization frequency of endophytes in various organs of *Butea superba* in monsoon at three places

Place	Total segments plated	Segments infested with fungi	Fungi isolated from the segments	Colonization frequency (CF%)	CF% in leaf	CF% in petiole	CF% in stem
Belpahari	75	72	72	96%	96%	100%	92%
Chilkigarh	75	74	74	98.64%	100%	96%	100%
Godapiasal	75	70	70	93.32%	84%	96%	100%
Total	225	216	216	96%	93.33%	97.33%	97.33%

Table 6. Endophytic fungi isolated from leaf (L), petiole (P) and stem (S) segments of *Butea superba* from different localities during monsoon

Isolated Endophytic fungi	Total isolates	Belpahari			Chilkigarh			Godapiasal		
		L	P	S	L	P	S	L	P	S
Alternaria sp.	04	0	0	0	0	2	2	0	0	0
Arthrinium sp.	14	1	4	3	2	2	0	2	0	0
Aspergillus sp.	07	0	0	0	1	2	4	0	0	0
Chaetomium sp.	22	0	0	0	4	4	4	1	8	1
Colletotrichum sp.	01	1	0	0	0	0	0	0	0	0
Fusarium sp.	123	16	13	16	13	7	6	14	14	24
Lasiodiplodia sp.	11	0	0	0	4	6	1	0	0	0
Mucor sp.	01	0	0	0	0	0	1	0	0	0
Mycelia sterilia	17	6	4	0	1	1	1	4	0	0
Nigrospora sp.	03	0	0	0	0	0	3	0	0	0
Penicillium sp.	02	0	0	0	0	0	2	0	0	0
Unidentified	09	0	2	4	0	0	1	0	2	0
Verticillium sp.	02	0	0	0	1	0	0	0	0	1
Total	216	24	23	23	26	24	25	21	24	26

Table 7. Diversity indices and species richness of endophytic fungi in *Butea superba* from Belpahari (Bel), Chilkigarh (Chil) and Godapiasal (God) during winter, summer and monsoon

Parameters	Winter			Summer			Monsoon		
	Bel	Chil	God	Bel	Chil	God	Bel	Chil	God
Species richness	5	6	3	6	7	6	5	12	6
Individuals	53	40	28	38	32	21	70	74	71
Simpson diversity	0.4763	0.575	0.4005	0.5956	0.6484	0.6712	0.5457	0.8181	0.4388
Shannon-Wiener index	0.9327	1.106	0.7119	1.259	1.444	1.409	1.081	2.015	0.9274
Evenness	0.5083	0.5036	0.6793	0.5869	0.6055	0.6817	0.5896	0.6253	0.4213
Fisher-alpha diversity	1.354	1.958	0.8516	2.004	2.765	2.806	1.232	4.059	1.563

Table 8. Comparison of isolated endophytic fungi from *Butea superba* in three seasons from three localities

Localities	Fungi isolated in various seasons from different places in *Butea superba*			
	Winter	Summer	Monsoon	Total
Belpahari	53	38	72	163
Chilkigarh	40	32	74	146
Godapiasal	28	21	70	119
Average	40.33	30.33	72	142.66

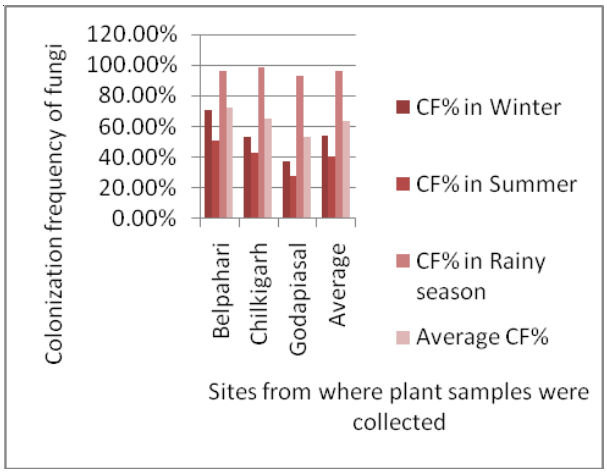

Fig. 1. Comparison of colonization frequency(CF) of isolated endophytic fungi

mum number of endophytes were found in stem (80%) and minimum in leaf (18.66%). Suryanarayanan and Rajagopal (2000) isolated 963 fungi from bark sample of 10 tropical tree species in southern India. Banerjee *et al.* (2010a) reported 14 endophytic fungal genera in 3 medicinal herbs. Only 6 different fungal genera and few unidentified genera with sterile mycelia were found. Collado *et al.* (1999) showed geographical and seasonal influences on the distribution of fungal endophytes. Among all isolated fungi of 3 different locations, *Fusarium* sp. was maximum in number. In Chilkigarh species diversity was more than other regions. Shannon-Wiener index was maximum in Chilkigarh forest (1.106) and Shimpson's diversity (0.575) was also maximum. Here, the maximum diversity of isolated endophytic fungi was in plants of Chilkigarh region

In summer study of *Butea superba,* the total tissue segments were 225 in 3 different regions, out of these 91 segments have been infested with endophytic fungi and 91 fungi have been isolated from them, i.e. CF% was 40.44%. Here in Belpahari CF% was maximum (50.64%) and in Godapiasal was minimum (28%). Out of various tissues, stem showed the maximum CF% (64%) for inhabitation of endophytes. In Chilkigarh area stem CF% was 96% and In Belpahari and Godapiasal also it was 48% each in stem that was maximum. 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. The maximum number of fungal genera was reported in Chilkigarh. Simpson's diversity and Shannon-Wiener index was maximum in Godapiasal (0.6712) and Chilkigarh (1.444) respectively. From the graph it was found that highest diversity of isolated endophytic fungi was in plants of Chilkigarh.

In monsoon study of *Butea superba*, total segments plated were 225 and segments infested with fungi were 216 i.e., colonization frequency were 96%. In Belpahari CF percentage was 96%, in Chilkigarh 98.64%, maximum and in Godapiasal it was 93.32%, was minimum. Leaf CF%=93.33%, petiole=97.33% and in stem CF was 97.33%. In leaf CF% was least and in petiole and stem it was equal. Total isolates were 216, and among which *Fusarium sp.*, greatest in number, was more or less equally distributed in all plant segments of all three places. *Colletotrichum sp*. was least in number, only one in leaf segment of Belpahari forest area. Total taxa were 5, 12 and 6 in Belpahari, Chilkigarh and Godapiasal respectively. Simpson's diversity was maximum in Chilkigarh (0.8181) and minimum in Godapiasal (0.4388). Shannon-Wiener index was also maximum in Chilkigarh (2.015) and minimum in Godapiasal (0.9274). Dominance index was maximum in Godapiasal (0.5612). Fisher alpha index was maximum in Chilkigarh (4.059). From the graph it was observed clearly that the highest diversity of

Table 9. Comparison of colonization frequency (CF%) of isolated endophytic fungi from *Butea superba* in three seasons from three localities

	Colonization frequency(CF) of fungi in various seasons from different places in *Butea superba*			
Localities	Winter	Summer	Monsoon	Average
Belpahari	70.66%	50.64%	96%	72.43%
Chilkigarh	53.33%	42.64%	98.64%	64.87%
Godapiasal	37.33%	28%	93.32%	52.88%
Average	53.77%	40.42%	95.98%	63.39%

isolated endophytic fungi was found in plants of Chilkigarh. In monsoon the maximum average number of isolated endophytic fungi and maximum colonization frequency was in plants of Belpahari. 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 oristic diversity, presence of relatively open canopies, highly variable annual rainfall and dry-season ground res Murali *et al*. (2007).

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 40, 30 and 72 respectively. Fungi isolated from Belpahari, Chilkigarh and Godapiasal were 163, 146 1nd 119 respectively. 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. Belpahari showed highest isolation and then Chilkigarh.

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