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A Relative Study of Endophytic Fungi from a Lianas Plant-Bauhinia vahlii During Summer and Monsoon Collected from Few Sites of West Bengal

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ABSTRACT

A relative study of endophytic fungal association with a lianas *Bauhinia vahlii* from different forest sites of West Bengal and their diversity during summer and monsoon was carried out. Samples were collected randomly in two seasons. Endophytic fungal isolates were identified based on mycelia shape and texture and colony formation; sexual and asexual reproductive structures and their characters; nature of spores and their attachment; various cultural conditions. A total of 311 endophytes were isolated from 304 various samplesegments. Colonization frequency is 66.66% in summer and 68.41% in monsoon. Maximum were under the class Sordariomycetes and Deuteromycetes. Majority of the endophytic isolates were from the plant segments of the leaf samples. The fungal isolates belong to the genera as many as 21, along with few unidentified fungi and few sterile mycelia. *Beltrania* sp., *Lasiodiplodia* sp., *Pestalotiopsis* sp., *Fusarium* sp., *Cylindrocladium* sp. were found most abundantly out of all isolated fungal endophytes. Maximum endophytic fungi were isolated from leaf tissues. Shannon-Weiner and Simpson's diversity indices show rich diversity of endophytic isolates in monsoon. The indices suggest even and uniform distribution of different species.

Keywords: Endophytes, Diversity, Lianas, Fungi, Bauhinia.

INTRODUCTION

Endophytic fungi are microorganisms that live within the inner tissue of plants without causing apparent symptoms (Wilson 2000).Carrol (1988) reported that endophytes live without any symptoms and sometimes systematically within the plant tissues. 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 (Petrini 1991). Microorganisms that colonize internal plant tissues without causing any diseases symptoms or apparent injury are called endophytes (Bacon and White 2000). Many fungal, bacterial, actinomycetean members are endophytes but most frequently isolated endophytes are fungi (Strobel 2002). They have been found infested with every plant species investigated so far. It is believed that plants from unique environ-

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mental settings and which are endemic are likely to accommodate distinct endophytic microorganisms as well as microorganisms making novel bioactive products (Strobel and Daisy 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 *Solanummelongena* 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).

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). They protect the plants against pests. They also enhance the defense mechanisms of host plant against unfavorable environments. Endophytic fungi show considerable antibacterial and antifungal activity (Jena and Tayung 2013). Various antifungal agents have been explored, but the control of many of the fungal diseases has not been achieved.Various biologically active natural metabolic products synthesized by microbial endophytes in association with their plant hosts are well classified (Firakova *et al.* 2007). Chen *et al*. (2014) isolated cytochalasins from cultures of an endophytic fungus. These bioactive products are of immense role in modern civilization. Salvianolic acid C is a hydrophilic phenolic acid consisting of two units of tashinol and one unit of caffeic acid, which is capable for the encouraging activities on cardiovascular and cerebrovascular diseases (Li *et al*. 2016). The fungus *Blastomyces* causes the disease blastomycosis (US Department of Health and Human Services 2017).

The goal of the study was to identify the fungal endophytic communities in leaves, petioles and stems of *Bauhinia vahlii* The objectives were to: Isolate the endophytic fungi, determine the diversity of endophytic fungi, compare the endophytic fungal isolates and their diversity pattern in relation to different forest regions and two seasons in the plant and to determine host organ specificity of fungal endophytes.

MATERIALS AND METHODS

The study was conducted in West Medinipur and Jhargram districts of West Bengal, India. The districts are situated in between the latitude of 22°25′ to 22°57′ North and longitude of 87°11′ 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. The lianas plant *Bauhinia vahlii* (Family- Papilionaceae) was selected from 3 different forest areas for present study. Plant samples (leaves, stems, petioles) were collected randomly from mature, healthy, disease-free plants from each location during summer and monsoon. The samples immediately after collection were kept in zipper-lock plastic bags, brought to the laboratory and stored at 4 ^{o}C within 2-3 hours of collection until isolation procedure was accomplished. 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, 70% ethanol for 20 sec. Finally, samples were rinsed with sterile distilled water for 3 times, then allowed to surface dry near flame of spirit lamp under sterile condition. 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. 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 potato dextrose agar

(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. 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 and Hunter 1998, Ellis Martin and Ellis Pamela 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 ×100 using the formula outlined by Hata and Futai: CF = (N_{col}/N_t×100, 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 allendophytes ×100. Dominant endophyte percentage (D) = N_i/N_s ×100, where N_i = percentage of colony frequency of individual endophytes, N_s = percentage of colony frequency of all endophytes. Using PalaeontologicalStatistics software package (PAST) (Hammer *et al*. 2001), following diversity indices were calculated: 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. 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 i^{th} species or kinds and measured by = n/N, N = total number of individuals of all kinds, n_i= number of individuals of i^{th} species, In = log to the base 2. 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.

RESULTS AND DISCUSSION

In summer, a sum of 150 tissue segments among 225 was infested with fungal endophytes and 153 were isolated. Mean colonization frequency (CF) was 66.66% and endophytic fungal members inhabited on petiolar stalk was 82.66% (Tables 1 and 2). CF is maximal in the plant of Belpahari (78.66%) and lowest in the host in Godapiasal (54.66%). As many as 12 genera of fungi with few unidentified and imperfecti on fungi were screened. The greatest figure of endophytic fungi was screened from the plant of Belpahari (60). Most of the endophytic fungi were colonized in petioles (82.66%). Leaf shows intermediate colonization frequency (62.66%) and stem showed minimum (54.66%). Earlier workers also noted and reported the tissue specificity of fungal endophytes (Banerjee *et al.* 2009, Raviraja 2005). Raviraja (2005) analyzed all the findings with the help of PAST statistical software. Species diversity of all isolated endophytic fungi was measured by the formulae, Simpson's diversity index, Shannon-Wiener index, Fisher alfa index, Manhinif index. *Bauhinia* sp. from Godapiasalexhibited the greatest Simpson's diversity (0.85) with highest Shannon-Weiner exponent (2.067). Highest Fisher-Alfa exponent (3.681) was in plants of Chilkigarh. All these indices indicate great species specificity of endophytes. In the present study *Lasiodiplodia* sp., *Beltrania* sp., *Fusarium* sp., *Chaetomium* sp., *Penicillium* sp. were the dominant fungal genera. From the graph it was found that the diversity of isolated endophytes was more or less equal in plants of three places and none of place

Table 1. Colonization frequency of endophyte in various organs of *Bauhinia vahlii* in summer at three places.

Place	Total segments	Segments infested with	Fungi sisolated from the	Colonization frequency (C F%)	CF % in leaf	CF % in petiole	CF % in stem
Belpahari	75	59	60	78.66%	84%	96%	56%
Chilkigarh	75	50	52	66.66%	56%	96%	48%
Godapiasal	75	41	41	54,66%	48%	56%	60%
Total	225	150	153	66.66%	62.66%	82.66%	54.66%

Table 2. Endophytic fungi isolated from leaf (L), petiole (P) and stem (S) segments of *Bauhinia vahlii* from different localities during summer.

Isolated endophytic fungi	Total isolate	Belpahari			Chilkigarh			Godapiasal		
		L	P	S	L	P	S	L	P	S
Arthrinium sp.	02	1	0	0	0	1	0	0	0	0
Aspergillus sp.	05	0	1	0	3	0	0	0	0	1
Beltrania sp.	26	5	9	1	4	5	2	0	0	0
Chaetomium sp.	10	0	0	3	0	3	0	0	4	0
Curvularia sp.	02	0	0	0	0	0	0	0	0	2
Diplodia sp.	03	1	2	0	0	0	0	0	0	0
Fusarium sp.	15	1	1	0	2	5	1	2	1	2
Lasiodiplodia sp.	56	11	9	9	3	8	6	2	5	3
Mucor sp.	01	0	0	0	0	1	0	0	0	0
Mycelia sterilia	14	1	2	1	1	3	2	1	3	0
Nigrosporasphaerica	02	0	0	0	0	0	0	0	1	1
Penicillium sp.	10	1	0	0	0	0	1	6	0	2
Unidentified	04	0	1	0	1	1	0	0	0	1
Verticillium sp.	03	0	0	0	0	0	0	0	0	3
Total	153	21	25	14	14	27	12	11	14	15

Table 3. Colonization frequency of endophytes in various organs of *Bauhinia vahlii* in monsoon at three places.

Place	Total segments plated	Segments infested fungi	Fungi isolated from the segments	Colonization frequency (CF %)	CF % in leaf	CF % in petiole	CF % in stem
Belpahari	75	45	48	60%	84%	40%	56%
Chilkigarh	75	44	45	58.64%	100%	12%	64%
Godapiasal	75	65	65	86.60%	92%	92%	76%
Total	225	154	158	68.44%	92%	48%	65.33%

shows maximum diversity (Figs. 1-5). But plants of Godapiasal show the highest colonization frequency in summer.

In monsoon, it was observed that the host lianas had a greater association of endophytic fungi. Total digits of 154 tissue specimens among 225 were associated with endophytic fungi and 158 fungi were isolated. Mean colonization frequency was 68.44% and foliar leaves of possessed greater number of fungal endophytes (92%). CF was highest in the

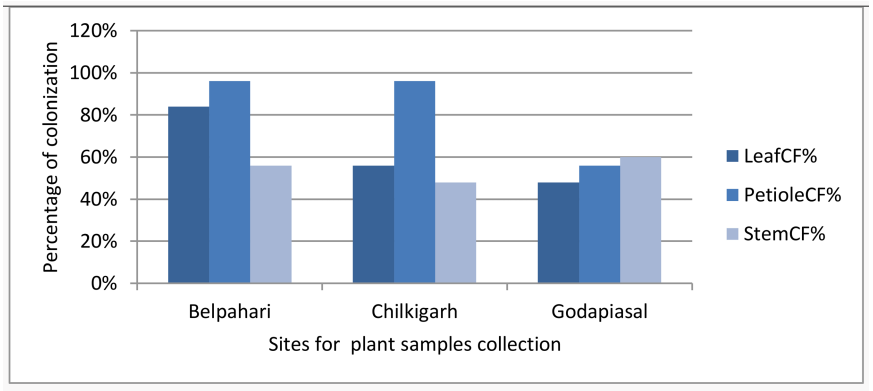

Fig. 1. Graph showing comparison of colonization frequency in various organs in summer.

Table 4. Endophytic fungi isolated from leaf (L), petiole (P) and stem (S) segments of *Bauhinia vahlii* of 3 localities in monsoon.

Isolated endophytic fungi	Total isolates	Belpahari			Chilkigarh			Godapiasal		
		L	P	S	L	P	S	L	P	S
Acremonium vitis	2	0	0	0	2	0	0	0	0	0
Alternaria sp.	6	0	1	1	4	0	0	0	0	0
Arthrinium sp.	14	1	1	3	2	0	0	0	5	2
Arthrobotrys sp.	22	0	0	0	0	0	0	20	2	0
Beltrania sp.	29	16	1	0	9	0	2	0	1	0
Fusarium sp.	17	0	2	2	1	1	4	1	6	0
Lasiodiplodia sp.	15	2	1	3	2	0	1	1	5	0
Mycelia sterilia	7	3	0	0	1	0	3	0	0	0
Nigrospora sphaerica	8	0	0	2	0	1	1	0	2	2
Papularia sphaerosperma	1	0	0	0	1	0	0	0	0	0
Penicillium sp.	3	0	3	0	0	0	0	0	0	0
Pestalotiopsis sp.	12	0	1	4	4	0	1	0	1	1
Torula sp.	2	0	0	0	0	0	0	0	0	2
Unidentified	5	1	0	0	0	1	0	1	1	1
Verticillium sp.	15	0	0	0	0	0	4	0	0	11
Total	158	23	10	15	26	3	16	23	23	19

host of Godapiasal (86.60%) and lowest in the host of Chilkigarh, 58.64%. Sum figure of 13 genera of isolated fungi with few unidentified genera and few mycelia sterilia were screened. Present investigation also shows the greatest assemblage of fungal endophytes in foliar leaf parts of the host of all 3 regions, 84%, 100% and 92% in the host plant respectively. Earlier it has been reported that colonization fungal endophytes was much greater in foliar leaf parts rather than stem parts of few medicinally important plant host in tropical regions (Raviraja 2005, Banerjee *et al.* 2006). It was observed and found that endophytic fungi has a tissue specificity i.e. leaves contain maximum number of endophytic fungi that tallies with the finding of other scientists (Banerjee 2011). It is clear that fungal assemblage is the lowest in petiolar parts. The principal genera of fungal endophytes screened are *Beltrania* sp., *Arthrobotrys* sp., *Fusarium* sp., *Lasiodiplodia* sp., *Verticillium* sp., *Pestalotiopsis* sp., *Arthrinium* sp. (Tables 3 and 4). Maximum were under the class Sordariomycetes and Deuteromycetes. Mean colonization frequency (CF) was in foliar leaves, petiolar parts and stems and figures are 92%, 48% and 65.33% respectively.

Diversity richness and species richness of fungal isolates were investigated in various tissues of the host species (Table 5). To determine the diversity indexes PAST software, version-1.89 was used. Greatest

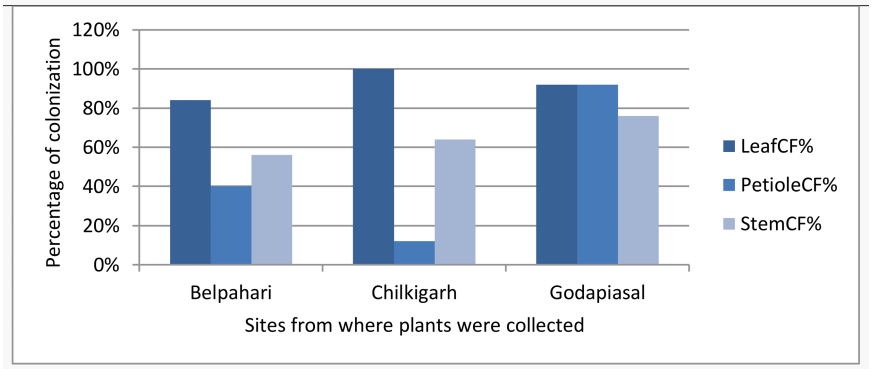

Fig. 2. Graph showing comparison of colonization frequency (CF) in various organs in monsoon.

Table 5. Diversity indices and species richness of endophytic fungi in *Bauhinia vahlii* from Belpahari (Bel), Chilkigarh (Chil) and Godapiasal (God) during summer and monsoon.

Paramater	Summer			Monsoon		
Sp. richness	10	10	10	10	12	10
Individuals	60	52	40	48	45	65
Simpson diversity	0.6922	0.8062	0.85	0.8186	0.8751	0.817
Shannon-Wiener index	1.564	1.89	2.067	1.998	2.267	1.959
Evenness	0.478	0.6617	0.7903	0.7373	0.8046	0.7094
Fisher-alpha diversity	3.427	3.681	4.28	3.843	5.354	3.3

Fig. 3. Plate culture of fungi isolated from *Bauhinia vahlii*:1.*Nigrospora sphaerica,* 2. *Lasiodiplodia* sp., 3. *Beltrania* sp., 4. *Pestalotiopsis* sp., 5. *Penicillium* sp.

Shannon-Wiener exponent (2.267) was observed in the host from Chilkigarh site and with maximal Simpson's diversity (0.8751) at the same area. It indicates great species specificity. Endophytic populations residing in a specific host plant may be universal that is very often regarded as host specificity (Carroll 1988, Petrini 1996, Stone *et al.* 2000). The result showed the richness of plant species in endophytic fungi. Banerjee *et al*. (2006) isolated different endophytic fungi from Vitex negundo in Mednipur, West Bengal, India. Here, from the graph it was observed clearly that the highest diversity of isolated endophytic fungi was found in plants of Godapiasal.

CONCLUSION

There is a diverse groups of endophytes in lianas plant found from my study. Majority has been identified with some unknown genera and some mycelia sterilia.

Table 6. Comparison of isolated endophytic fungi from *Bauhinia vahlii* in two seasons from three localities.

Localities	Monsoon	Summer	Total
Belpahari	48	60	196
Chilkigarh	45	52	194
Godapiasal	65	41	192
Average	52.66	51	194

There is no host and region specificity of endophytes and but they have organ and tissue specificity of a particular host. The plant of *Bauhinia vahlii* shows that it possess many known and common endophytes with some unknown members. The woody lianas plants of all places were associated with numerous fungal endophytes making a mutualistic assemblage. Endophytes are assemblage maximum in monsoon than summer; most probably microclimate with shady, damp, moderate temperature play important role in more association of endophytes (Tables 6 and 7).

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Table 7. Comparison of colonization frequency (CF%) of isolated endophytic fungi from *Bauhinia vahlii* in two seasons from three localities.

Localities	Summer	Monsoon	Average
Belpahari	78.66%	60%	65.95%
Chilkigarh	66.66%	58.64%	66.16%
Godapiasal	54.66%	86.60%	68.42%
Average	66.66%	68.41%	66.84%

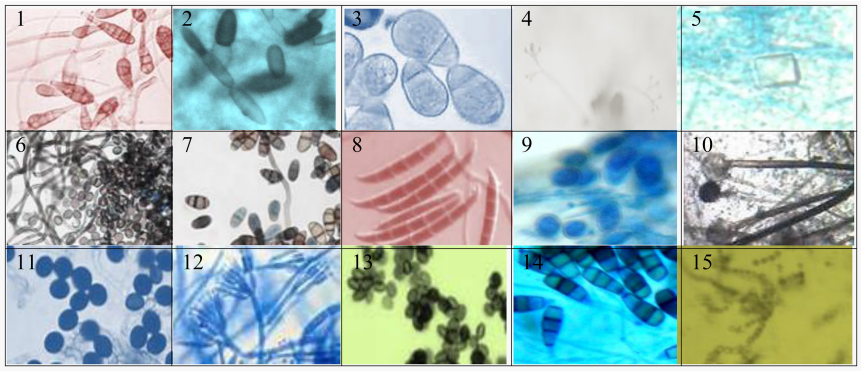

Fig. 4. Microscopic pictures of few endophytic fungal isolates-1. *Alternaria* sp., 2. *Arthrinium* sp., 3. *Arthrobotrys* sp., 4.*Verticillium* sp., 5. *Beltrania* sp., 6. *Chaetomium* sp., 7. *Curvularia* sp., 8. *Fusarium* sp., 9. *Lasiodiplodia* sp., 10. *Mucor* sp., 11. *Nigrospora* sp., 12. *Penicillium* sp., 13. *Papularia* sp., 14. *Pestalotiopsis* sp., 15. *Torula* sp.

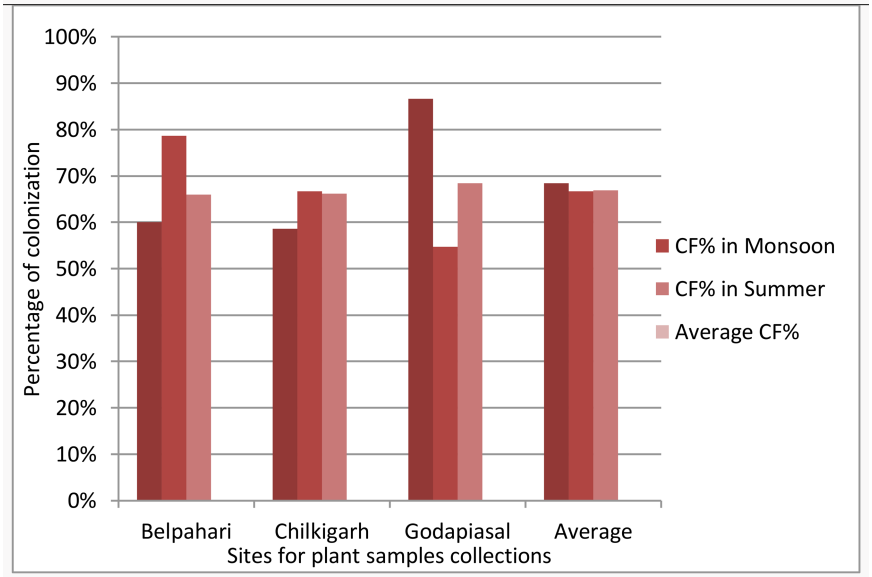

Fig. 5. Comparison of colonization frequency of isolated endophytic fungi.

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