Isolation of Endophytic Fungi from Selected Bamboo Species of Arunachal Pradesh, India

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Abstract- The present study attempts to isolate and identify the number of endophytic fungal association in three species of bamboo leaves; *Dendrocalamus sikkimensis* (DS), *Bambusa polymorpha* (BOP) and *Phyllostachys bumbusoides* (PB). The endophytic fungi was isolated from the plant tissue following the protocol of Suryanarayanan *et al.*, 1998. A total of 196 endophytic fungi were isolated and identified up to genus level. Among all the fungal isolates, *Xylaria* and *Nigrospora* sp. showed highest level of colonization frequency (i.e. 18%). *Xylaria* sp. was more dominant in BOP, whereas, DS and PB displayed deviation in terms of fungal dominance with sp2 (sterile) and *Nigrospora* sp. to be dominant respectively. The isolation of endophytic fungi from healthy mature leaves of host plant suggest that the fungi residing inside them is asymptomatic and may be beneficial to their host, as they are found to protect their host against domestic herbivores, pest and pathogens. The study opens the gate for further biological analysis of endophytic fungi associated with bamboo creating a genetic resources for future researchers.

Index Terms- Bamboo endophytes, Bambusicolous fungi, Arunachal pradesh, *Xylaria* sp.

I. INTRODUCTION

India being the second largest producer of bamboo worldwide, has the richest diversity with 23 genera and 125 species, both indigenous and exotic (Verma and Bahadur, 1980). The North Eastern part in India alone harbors major diversity of bamboo consisting of 19 genera and 78 species (Hore, 1998) and Arunachal Pradesh consisting of 57 species (Bhuyan *et al.*, 2007) is richest in the country as far as the bamboo resources are concerned. This region of India represents existing bond for both indigenous knowledge on utilization and management of bamboo species, which goes along with the traditional uses.

Endophytes are asymptomatic microorganism colonizing the living tissue of plants (Wilson, 1995) and believed to be protecting its host plant from both abiotic and biotic stresses (Rodriguez *et al.*, 2008). They mostly belong to ascomycetes and anamorphic fungi (Arnold, 2007), representing an important part of fungal diversity and are known to affect the diversity and structures of plant communities (Krings *et al.*, 2007) with promoting the growth of its host plant (Omacini *et al.*, 2001).

Review on literature related on bamboo-associated fungi reveals nearly about 1100 species belonging to 228 genera of bambusicolous fungi (Hyde *et al.*, 2002a, b). Tanaka and Harada (2004) mentioned some important bamboo-associated fungi from Hino and Katumoto records. Among the bambusicolous fungi, the described population of endophytic fungi of bamboo is very limited. The largest diversity of bamboo endophytes recorded in Asia were roughly 500 species and about 38% were recorded from Japan (Hyde *et al.*, 2002).

Owing to global diversified distribution of bamboo in tropical, subtropical and temperate regions, its micro-fungi (endophytes) are widely studied. Most bamboo associated fungal records were based on its symptoms and morphology, thus, requiring a more extensive study on its molecular taxonomy. Also, majority of taxa of endophytes related with bamboo species have been recorded from other parts of Asia like China and Japan, while little has been known from India and South America (Bhagobaty and Joshi, 2011).

Arunachal Pradesh, being one of the biodiversity hot post of India has the potential to create genetic resources based on endophytic fungi from bamboo and provide a plethora for novel substances which can be of probable use in modern medicine, agriculture, antimitotic, immune suppressants and anti-cancer compounds (Strobel and Daisy, 2003). Till date, there has been very limited study on fungal endophytes of Arunachal Pradesh. Thus, investigation on the endophytes of bamboo will help in broadening the knowledge on untapped endophytic fungal resources.

II. MATERIALS AND METHODS

2.1 Collection site

The three bamboo species; *Dendrocalamus sikkimensis* (DS), *Bambusa polymorpha* (BOP) and *Phyllostachys bumbusoides* (PB), were collected from two different regions of the state (Fig. 1). Bamboo species DS and BOP were collected from Bamboo setum in Chessa Van Vigyan Kendra in Papum Pare Dist., and PB was collected from Ziro, Lower Subansiri Dist (Fig 1.). From each of the selected bamboo species, healthy mature leaves were selected and collected in a sterile collection bag.

2.2 Isolation of endophyte

The fresh mature healthy leaves of bamboo species were collected in sterile collection bag from different sites of Arunachal Pradesh and processed for isolation of fungal endophytes within 24 to 36 hours of collection (Fisher *et al.*, 1987; Suryanarayanan *et al.*, 1998). The leaf samples were washed in running water for any debris and dirt and one hundred tissue segments of 0.5 cm^2 were excised from each bamboo species. Each leaf tissue segments were excised so as to include apical, middle and basal portion of

the leaf including the lamina. The tissue samples were then surface sterile following the protocol of Suryanarayanan *et al.,* 1998. The tissue segments were dipped in 70% ethanol for 5 second followed by 4% NaOCl for 90 seconds and finally rinsed with sterile distilled water for 10 seconds for surface sterilization. The segments were then inoculated in freshly prepared PDA (potato dextrose agar) media with desired antibiotics (Chloramphenicol). Total of 10 petri dishes were inoculated for each bamboo species where, each petri plates were inoculated with 10 tissue segments and kept for incubation at 26 ± 2°c under 12 hours light and 12 hours dark period for 4 weeks (Suryanarayanan, 1992).

2.3 Identification of endophytic fungi

The endophytic fungi grown from the tissue segment were isolated to make pure culture and identified both morphologically and microscopically following the standard manuals described by Barnett and Hunter 1972; Wei, 1979; Carmichael *et al.,*1980. The microscopic observations were made by observing the slide culture under the microscope and photographs were taken with the help of *Lica* DM500 (Fig 3.). The sterile isolates were given codes based on culture characteristics such as growth rate, colony surface texture and hyphal pigmentation (Suryanarayanan *et al.,* 1998) and were expected to represent different taxonomic species (Bills and Polishook, 1994).

2.4 Statistical analysis

For data analysis, Colonization Frequency (CF) of each endophytes and Isolation Rate were calculated.

$$CF\% = \frac{No.of segments colonized by each endophytes}{Total no.of segments observed} \times 100$$

$$\text{Isolation Rate (\%)} = \frac{The\ number\ of\ fungi\ obtained\ from\ segmenst}{Total\ numbers\ of\ segments\ inoculated} \times 100$$

Simpson index (D), Shannon-Wiener's diversity (HS) and Margalef's species richness index (R1) (Shannon and Weiner, 1963; Yuan *et al.,* 2010; Maheshwari and Rajagopal, 2013) were used to assess and quantify endophytic fungal diversity in host plants.

Simpson's index of Diversity was calculated using the formula: 1-D

$$D = \sum_{i=1}^{s} \frac{N(N-1)}{n(n-1)}$$

Where,

n = the total number of organisms of a particular species and N = the total number of organisms of all species.

Shannon-Wiener Diversity index (HS) was calculated using the following formula:

$$H_s = -\sum_{i=1}^{s} (Pi)(lnPi)$$

Where,

Hs= Diversity in a sample of S species or kinds,

S= Number of species in the sample,

Pi= Proportion of total sample represented by species (i). Where, i is number of individuals of species.

N= Total number of individuals of all kinds, Ni is the number of individuals of species i and ln is log to base 2.

Margalef's Species Richness R1 was calculated using the following formula:

$$R1 = \frac{(S-1)}{\ln(N)}$$

Where, S = total number of species and N = the total number of isolates of all species.

III. RESULTS

In the present study a total of 196 endophytic fungi were isolated from healthy matured leaves of three bamboo host plant and identified up to the genus level. The fungal endophytes isolated are characterized into 11 morphotypes of fungi depending on colony morphology and microscopic structures (Fig 3). Out of 11, three fungal genus are characterized as sterile, as no spore formation were seen. All the fungal endophytes isolated belong to phylum Ascomycota. Of the total fungal isolates, 65 isolates belonged to Sordariomycetes genera, 59 to Hypomycetes, 6 belonged to Coelomycetes, 2 to Eurotiomycetes and 2 Ascomycetes.

Among all the fungal isolates, *Xylaria* and *Nigrospora* sp. showed highest level of isolation rate (i.e. 18%) and *Penicillium* sp. and *Chaetomium* sp. showed lowest isolation rate (0.66%) (Table 1; Fig 2). *Xylaria* sp. was more dominant in BOP, whereas, DS and PB displayed deviation in terms of fungal dominance with sp2 (sterile) and *Nigrospora* sp. to be more dominant respectively. The host sample *D. sikkimensis* showed highest level of species diversity depicting similar result with values 0.818, 1.85 and 1.739 in Simpson's index of diversity, Shannon diversity index and Margalef's Species Richness, respectively (Table 2). In contrast, *B. polymorpha* showed lowest value for diversity with values 0.488, 0.787 and 0.476 in Simpson's index of diversity, Shannon diversity index and Margalef's Species Richness respectively. The statistical analysis also explains that DS shows more evenness in terms of species distribution with the value of 0.794 (Table 2). The ubiquitous nature of endophytic fungi is depicted by the omnipresence of *Nigrospora* sp. and *Xylaria* sp. in all the three host plant showed in Table 1. Each host species were collected from different sites of Arunachal Pradesh and all the three host displayed different dominant fungi, suggesting that the association of endophytic fungi may be environmental and host dependent. The statistical analysis explains that DS is more diverse with higher species richness and evenness when compared with other two host plant (Table 1).

IV. CONCLUSION

In the present investigation, the versatility of colonization of fungi is limited as the endophytic fungal isolates such as *Xylaria* and *Colletotrichum* occur in wide range of plant host and in various geographical location, dominating the assemblage of plant tissues, thus creating a depression in overall endophytic diversity in a given ecosystem (Sudhakara *et al.,* 2016).

The result also corroborated with the previous findings, which has been isolated previously in bamboo tissues (Morakotkarn*et al.,* 2007; Shen *et al.,* 2012). Previous reports have also mentioned the species of endophytic fungi to be identified from Dothideomycetes and Sordariomycetes, isolated from tissues of bamboo species and their molecular diversity were analyzed based on ITS region of ribosomal DNA (Morakotkarn*et al.,* 2007; Shen *et al.,* 2012).

The leaves of all the host plant recruit similar type of endophytes such that the frequency of endophytes in leaves increases but not its diversity (Murali *et al.*, 2007). The scenario is so, because of the presence of multi-host endophytes such as *Colletotrichum* sp. and *Xylaria* sp. (Suryanarayanan, 2017). The statistical analysis of diversity indices shows that Shannon-Wiener Diversity is high (1.85) in *D. sikkimensis* than other bamboo species (Table 2). The value of Shannon diversity indices, Simpson's index and Margalef diversity indices shows increased evenness in bamboo species coded DS than other two bamboo host plants (Table 2). The higher level of evenness can be contemplated to show increase in fungal diversity.

The isolation of endophytic fungi from healthy mature leaves of host plant suggest that the fungi residing inside them is asymptomatic and may be beneficial to their host, as they were previously found to protect their host against domestic herbivores, pest and pathogens (Weber, 1981).

About 93% of the estimated fungal species are found in tropics and a stress is needed to explore the various ecological groups of fungi including endophytes to identify novel species of fungi (Manoharachary *et al.*, 2005). India has reported of about 27,500 fungal species which includes 15,500 from litter, 327 from dung of herbivore and 450 endophytes (Bhat, 2010) which is scanty number for a country with great multitude of geographical area supporting vast habitat. Endophytic fungi are gaining attention as they appear to be more innovative in terms of metabolic activity than soil fungi or fungi in association with algae (Schulz*et al.*,2002) in producing bioactive compounds. Very limited study has been done on endophytes associated with bamboo and its data relating to its diversity in India as a whole and Arunachal Pradesh in particular is scares. Most of the study on endophytes related to bamboo in done on other part of Asia like China and Japan. So, focusing on isolating fungal endophytes and study of its biological activity from one of the major hotspot (Myer *et al.*, 2000) area like Arunachal Pradesh ponder a great interest.

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Figure legends:
Fig 1. Map of Study area
Fig 2. Isolation rate of each endophytic fungi from some selected bamboo species of Arunachal Pradesh, India
Fig 3. Endophytic fungi from some selected bamboo species of Arunachal Pradesh, India:
A. *Arthrinium* sp. (a1) Microscopic structure of spores (a2) Colony morphology
B. *Chaetomium* sp. (b1) Microscopic structure of spores (b2) Colony morphology
C. *Colletotrichum* sp. (c1) Microscopic structure of spores (c2) Colony morphology
D. *Fusarium* sp. (d1) Microscopic structure of spores (d2) Colony morphology
E. *Nodulisporium* sp. (e1) Microscopic structure of spores (e2) Colony morphology
F. *Penicillium* sp. (f1) Microscopic structure of spores (f2) Colony morphology
G. *Xylaria* sp. (g1) Microscopic structure of spores (g2) Colony morphology
H. *Nigrospora* sp. (h1) Microscopic structure of spores (h2) Colony morphology

Table 1: Number of endophytic fungi isolated

Fungi	Segment of host plant used for isolation	DS	BOP	PB	Total no. of fungal strains	Isolation rate (%)
Xylaria sp.	Leaf	10	43	1	54	18%
Nodulisporium sp.	Leaf	6	3	2	11	3.66%
Colletotrichum sp.	Leaf	5	-	1	6	2%
Arthrinium sp.	Leaf	15	-	-	15	5%
Nigrospora sp.	Leaf	-	-	54	54	18%
Penicillium sp.	Leaf	2	-	-	2	0.66%
Fusarium sp.	Leaf	3	-	1	4	1.33%
Chaetomium sp.	Leaf	2	-	-	2	0.66%
Sterile sp.2	Leaf	13	-	-	13	4.33%
Sterile sp.4	Leaf	-	21	-	21	7%
Sterile sp.7	Leaf	-	-	15	15	5%
CF%		56	67	73		
Total no. of species		8	3	6		

Sample size: 100 leaf tissue segments (0.5 cm^2) from each bamboo species

Table 2: Dominance and species Richness of endophytic fungi in Bamboo species

Bamboo species.	Dominance_D	Simpson_1-D	Shannon_H	Evenness_e^H/S	Margalef
BOP	0.512	0.488	0.787	0.732	0.476
PB	0.575	0.425	0.826	0.380	1.162
DS	0.182	0.818	1.85	0.794	1.739

Note: Map is not for scale

Figure 1. Map of collection site

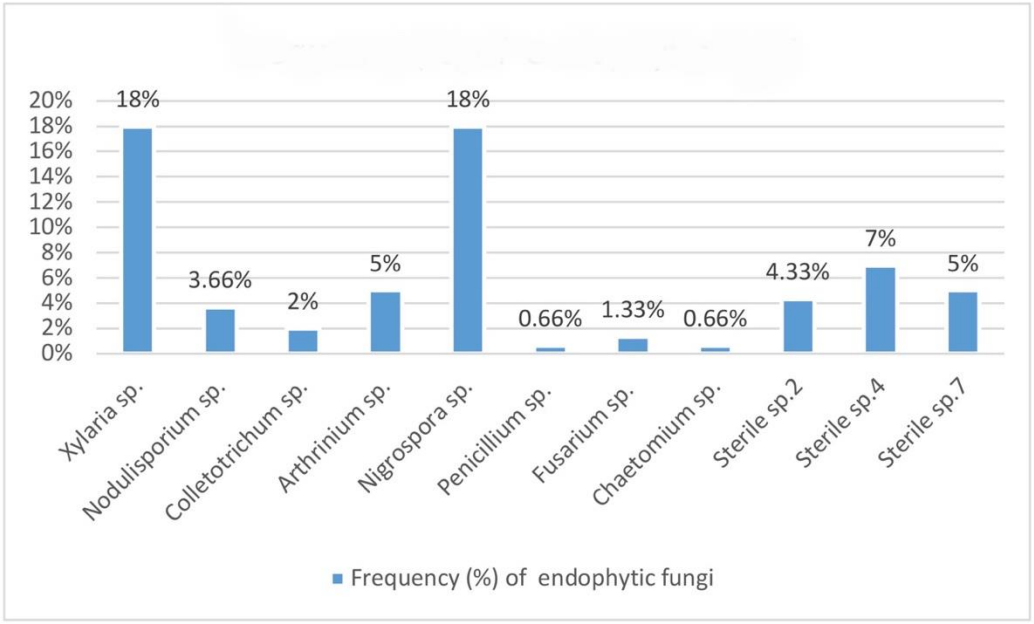

Figure 2. Isolation Rate of each endophytic fungi from some selected bamboo species of Arunachal Pradesh, India

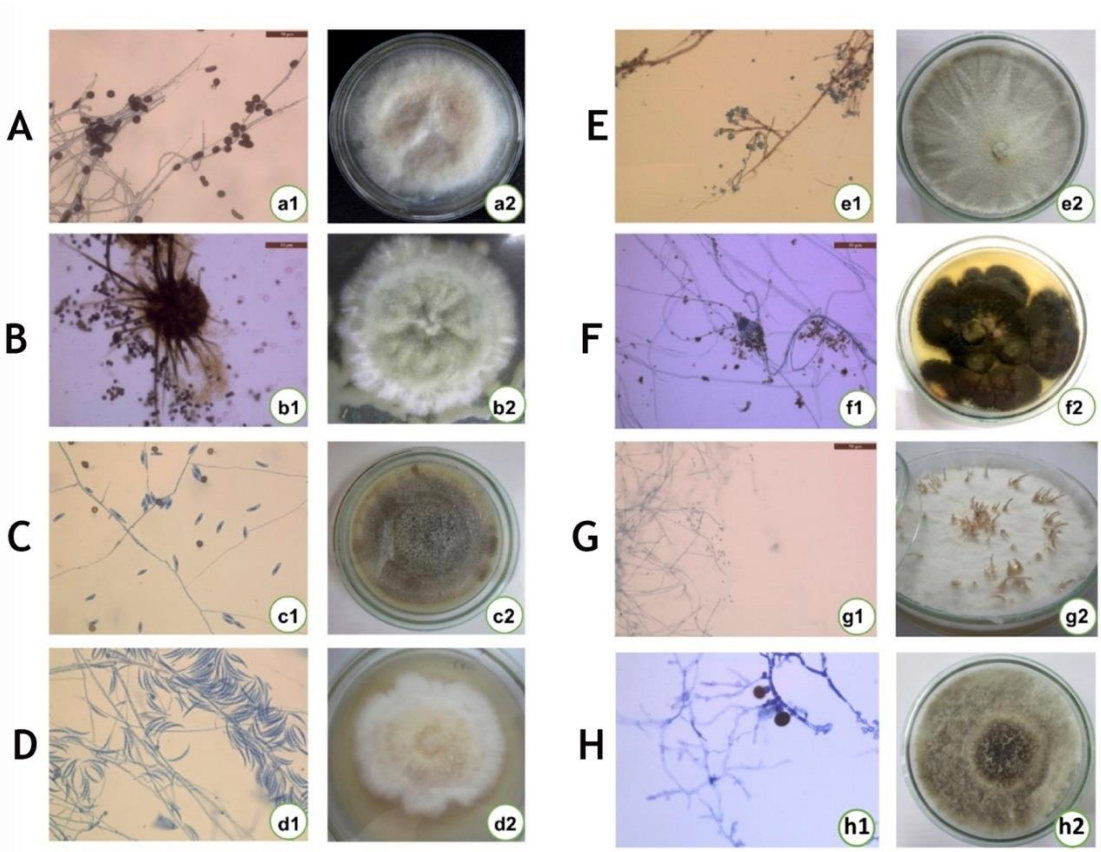

Figure 3. Endophytic fungi from some selected bamboo species of Arunachal Pradesh, India: A. *Arthrinium* sp. (a1) Microscopic structure of spores (a2) Colony B. *Chaetomium* sp. (b1) Microscopic structure of spores (b2) Colony C. *Colletotrichum* sp. (c1) Microscopic structure of spores (c2) Colony D. *Fusarium* sp. (d1) Microscopic structure of spores (d2) Colony E. *Nodulisporium* sp. (e1) Microscopic structure of spores (e2) Colony F. *Penicillium* sp. (f1) Microscopic structure of spores (f2) Colony G. *Xylaria* sp. (g1) Microscopic structure of spores (g2) Colony H. *Nigrospora* sp. (h1) Microscopic structure of spores (h2) Colony.