Antimicrobial endophytic fungal assemblages inhabiting bark of *Taxus baccata* L. of Indo-Burma mega biodiversity hotspot

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Received: 21 February 2008 / Accepted: 12 June 2008
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Abstract Fungal endophytes were isolated from inner bark of *Taxus baccata* L., an important source of potent anticancer drug taxol. Bark samples were collected from two locations of Arunachal Pradesh, India, part of the Indo-Burma mega biodiversity hotspot, during two seasons i.e. monsoon and winter. Altogether 77 fungal strains representing 18 genera were isolated from *T. baccata* bark during the present investigation. The colonizing frequency was recorded as 38.5% and the fungal community comprised of 78% of Hyphomycetes, 5.2% of Coelomycetes, 2.6% of Zygomycetes and Ascomycetes and 9.1% of sterile mycelia. Most common and frequently isolated genera were *Fusarium*, *Penicillium* and *Aspergillus*. Simpson and Shannon diversity indices indicated higher species diversity during monsoon than during winter seasons irrespective of the locations. The two locations harbored 5 to 37 endophyte species and the similarity index was low during winter and high during monsoon. Ethyl acetate extract of fermentation cultures of these fungi were tested for their antimicrobial activity against a panel of human pathogenic Gram-positive and Gram-negative bacteria and fungi. Fifteen fungal isolates out of the isolated strains displayed antimicrobial activity. An endophytic fungus, identified as *Fusarium sp.* displayed significant antimicrobial activity against all the test pathogens.

Keywords Fungal endophytes · *Taxus baccata* · Indo-Burma mega biodiversity hotspot · Antimicrobial activity

Introduction

Fungal endophytes even though very important are relatively unstudied group of microbes that colonize healthy plant tissues without causing any overt symptoms or apparent injury to the host [1]. They have been found in every plant studied so far and are expected to be present in virtually every plant on the earth [2, 3]. There are fewer studies related to the endophyte assemblages of woody plants than those of grass despite of their great diversity and abundance [4]. A few studies on endophytes of tropical plants as regards to their relative abundance, colonization and species composition have indicated their presence in several trees and shrubs [5, 6]. Studies suggest that endophytes in tropical forests may contribute substantially to fungal diversity and shape plant community dynamics [7].

Different works carried out so far regarding the role of endophytes in host plants indicate that they can stimulate plants growth, increase disease resistance, improve plant's ability to withstand environmental stresses and recycle nutrients [8]. Besides these, endophytes are also recognized as rich sources of bioactive metabolites of multifold importance [9, 10]. Strobel and Daisy [10] necessitated that plants growing in unique environmental setting having ethno medicinal uses, extreme age or endemism should be selected for these studies because they are expected to harbor novel endophytes that may produce unique metabolites having diversified applications. Moreover, it is believed that the

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healing properties possessed by the ethnomedicinal plants, as discovered by indigenous people, might be because of compounds produced by one or more specific plant-associated endophytes. There are several reports indicating the antimicrobial properties of fungal endophytes colonizing medicinal plants [11–13].

Taxus baccata L., a non-resinous Gymnosperm plant found distributed in the temperate regions of Eastern Himalayas, Arunachal Pradesh, part of Indo-Burma mega biodiversity hotspot, is of great importance because its leaf and bark are prime source of taxol—a potent anticancer drug [14]. In the Unani system of medicine, the extracts of bark and leaf of *Taxus baccata* are source of drug Zarnab, used for treating various disorders [15]. Local population of Arunachal Pradesh use *Taxus* bark for preparing ‘*Namkin chay*’ and use as medicine [14]. Endophytes harboring yew plant around the world have been reported to produce taxol and other bioactive metabolites [16–18]. Studies on fungal endophytes colonizing *Taxus* plant from this mega biodiversity hotspot part of the world, however, have not yet been reported. In this study, an attempt was made to study the diversity of endophytes colonizing bark of *Taxus* and to screen the antimicrobial producing strains.

Materials and methods

Location and climate of the study area

The plant materials were collected from two different locations of Arunachal Pradesh. The study was conducted at Dibang valley (2600 m above MSL) and Tawang valley (3200 m above MSL). The study areas were located between latitude 28°20′7″N and longitude 95°46′38″E at Dibang valley and between latitude 27°35′25″N and longitude 91°65′45″E at Tawang valley. In Dibang valley, the average annual rainfall ranges from 980 to 3700 mm, while the maximum and minimum temperatures range from 22 to 35°C and -2 to 14°C respectively. The average rainfall

ranges from 450 to 1650 at Tawang valley. The maximum and minimum temperatures vary from 15 to 28°C and -6 to 12°C, respectively.

Collection of samples

Sampling was done during two seasons i.e. monsoon and winter seasons. Barks were cut off from the selected healthy plants with an ethanol-disinfected sickle and placed in separate polythene bags to avoid moisture loss. The materials were transported to laboratory within 48 h and stored at 4°C until isolation procedures were completed.

Isolation of endophytes

The collected bark samples were washed thoroughly in distilled water before they were surface sterilized. Washed barks were cut into 5–6 cm pieces with a sterile knife and surface sterilized by immersing them sequentially in 70% ethanol for 3 min and 0.5% NaOCl for 1 min. Thereafter the pieces were rinsed thoroughly with sterile distilled water [2, 19]. The excess water was dried under laminar airflow chamber. Then, with a sterile scalpel, outer tissues were removed and the inner tissues were carefully dissected and placed on Petri-plates containing different mycological media and media amended with bark extracts. For preparation of bark extract, 250 gm bark/100 ml distilled water was brewed and the decoction was collected which was then added into the medium (Table 1). Sterptomycin (100 mg/l) was added to each medium in order to suppress bacterial contamination. The plates were sealed with parafilm and incubated in BOD incubator at 24±1°C for days until fungal growth appeared on the plates. After incubation the hyphal tips of the fungi growing out of the plated bark were removed with a fine sterile needle and transferred to freshly prepared potato dextrose agar slants. Tubes were incubated for 1–2 weeks and periodically checked for purity of the culture. The fungal isolates were observed under microscope and their morphological characters were noted.

Table 1 Recovery of endophytes from *Taxus baccata* bark on different media

Sample	Media*	Incubation Time (in weeks)	No. of colonies recovered/200 segments
Bark	PDA	3	10
	MEA	2	14
	WA	2-3	08
	PDA+BE	2	15
	MEA+BE	2	18
	WA+BE	1-3	12

PDA-Potato dextrose agar; MEA-Malt extract agar; WA-Water agar; BE-Bark extract

Sporulation was induced in non-sporulating endophytes by incubating them at different temperatures, alternate dark/light phase and by culturing them in media amended with bark extracts. The fungal isolates were identified based on their morphological and reproductive characters using standard identification manuals [20–22]. Cultures that failed to sporulate were recorded as sterile mycelia. All the isolates were maintained on PDA with 15% glycerol at -20°C in deep freezer at the Department of Botany, Gauhati University, Guwahati, India.

Fungal cultivation and metabolite extraction

The fungal endophytes were cultivated on Potato dextrose broth by placing agar blocks of pure culture (3 mm in diameter) of actively growing culture in 250 ml Erlenmeyer flasks containing 100 ml of the medium. The flasks were incubated in BOD shaking incubator for 3–4 weeks at $24\pm 1^{\circ}\text{C}$ with periodic shaking at 150 rpm. The culture was filtered through sterile cheesecloth to remove the mycelial mats. The liquid broth was collected and extracted with equal volume of ethyl acetate in a separating funnel by vigorous shaking for 10 min. The cell mass was separated and the solvent so obtained was collected. Ethyl acetate was evaporated and the resultant compound was dried with MgSO_4 and concentrated to yield the crude extracts. The crude extracts were then dissolved in Dimethyl sulphoxide (DMSO) for antimicrobial bioassay.

Evaluation of antimicrobial activity

Antimicrobial activity of the compound was tested against human pathogenic test organisms by agar cup diffusion method. The test organisms include three Gram-positive bacteria, *Bacillus subtilis* (Bs), *Staphylococcus epidermis* (Se) and *Staphylococcus aureus* (Sa), three Gram-negative bacteria *Klebsiella pneumoniae* (Kp), *Shigella flexneri* (Sf) and *Escherichia coli* (Ec) and two pathogenic fungi *Candida albicans* (Ca) and *Candida tropicalis* (Ct). The test bacterial pathogens were maintained on freshly prepared nutrient agar slants while the test fungi were cultured on Sabouraud's agar slants. All media were procured from Hi-Media, Mumbai, India. Nutrient agar plates were inoculated with 0.2 ml of overnight grown culture containing 1.0×10^9 cells. Similarly Sabouraud's agar plate was inoculated with 0.2 ml of cultured test fungi containing 1.0×10^9 cells. The plates were evenly spread out with a help of a sterile cotton swab. Then agar cups were prepared in the plates with a cork borer (7 mm in diameter). Each cup was then loaded with 100 μl of the crude extracts dissolved in DMSO. The control cup was filled with only DMSO. The plates were incubated at 37°C for 24 h and the zone of inhibition was

measured and compared with the control. Three replicates were maintained in each case. The magnitude of antimicrobial action was assessed by the diameter (mm) of inhibition zones and compared with co-assayed antibiotics Tetracycline as antibacterial and Fluconazole as antifungal agent.

Data analysis

The relative frequency of colonization (% CF) was calculated as the number bark segments colonized by a specific fungus divided by total number of segments plated $\times 100$ [23] and dominant endophytes were calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes $\times 100$ [24].

Jaccard's similarity coefficients were calculated for the host species based on the endophytic fungi colonized using the formula:

$$\text{Similarity Coefficient} = C/(A + B + C)$$

where A and B are total number of endophytic fungal species recovered from any two hosts and C is the number of fungal species in common.

Simpson's index of Diversity was calculated using formula:

$$1-D$$

$$\text{where } D = \frac{\sum n(n-1)}{N(N-1)}$$

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:

$$H_s = - \sum_{i=1}^S (P_i) (\ln P_i),$$

where

H_s – symbol for the diversity in a sample of S species or kinds

S – the number of species in the sample

P_i – relative abundance of i^{th} species or kinds measures, $= n_i/N$

N – total number of individuals of all kinds

n_i – number of individuals of i^{th} species

ln – log to base 2

Results and discussion

A total of 77 isolates belonging to 18 different genera were recovered from healthy inner bark of *Taxus baccata*

(Table 2). The endophytes were isolated using different mycological media as well as media amended with bark extract of the host plant (Table 1). Maximum endophytes were isolated using malt extract agar medium amended with bark extract (MEA+BE) and minimum on water agar medium (WA). The number of isolates obtained was higher when media were amended with *Taxus* bark extract. This indicated the presence of some growth stimulatory molecules in the bark that might have favored the growth of the endophytes. Similar observations were made where endophytes sporulate only when autoclaved host tissues were added to the culture media [25, 26]. The colonization frequency was 38.5%. The fungal endophytes composition include 2.6% of zygomycetes, 2.6% of ascomycetes, 5.2% of coelomycetes, 78% of hyphomycetes and 9.1% of sterile mycelia. The two locations differed in the percentage occurrence of endophytes during the two seasons. Dibang

and Tawang valley respectively harbored 48 and 19.5% of the total isolates during monsoon. During winter, however, just 22 and 10.4% of the total isolates could be respectively isolated from both the sites. In many instances, bark, twig and leaf samples collected during monsoon harbored more endophytes than during the dry season [5, 27]. Recovery of endophytes was more (70%) from Dibang than Tawang valley (30%). The occurrence of more endophytes in the Dibang valley may be due to the favorable environmental conditions with moist environment than the Tawang valley, which was comparatively drier. *Fusarium*, *Penicillium* and *Aspergillus* were the most common and frequently isolated fungal genera from both the locations during both the seasons. They appeared as the dominant genera. Such common occurrence of the genera *Aspergillus* and *Penicillium* have also been reported as endophytes from healthy tissues of *Melia azedarach* [28]. Further, during winter more

Table 2 Occurrence of endophytic fungi in *Taxus baccata* bark during different seasons growing at different altitudes of Arunachal Pradesh

Endophytic fungi	Monsoon		Winter		No. of Endophytes	CF*	Frequency endophytes of dominant
	Db	Tw	Db	Tw			
<i>Acremonium acremonium</i> .	2.0	–	–	–	2.0	1.0	2.60
<i>Alternaria alternata</i>	4.0	–	2.0	1.0	7.0	3.50	9.09
<i>Aspergillus versicolor</i>	2.0	1.0	1.0	–	4.0	2.0	5.19
<i>Aspergillus sp.</i>	–	1.0	–	2.0	3.0	1.50	3.90
<i>Chaetomium globosum</i>	2.0	2.0	–	–	4.0	2.0	5.19
<i>Cladosporium cladosporioides</i>	–	1.0	–	–	1.0	0.50	1.30
<i>Colletotrichum sp.</i>	1.0	–	2.0	–	3.0	1.50	3.90
<i>Curvularia lunata</i>	1.0	–	–	–	1.0	0.50	1.30
<i>Drechslera sp.</i>	–	1.0	–	–	1.0	0.50	1.30
<i>Fusarium avenaceum</i>	–	1.0	3.0	2.0	6.0	3.0	7.80
<i>Fusarium moniliforme</i>	2.0	–	–	–	2.0	1.0	2.60
<i>Fusarium sp.</i>	2.0	1.0	1.0	–	4.0	2.0	5.19
<i>Gliocladium sp.</i>	2.0	2.0	–	–	4.0	2.0	5.19
<i>Mucor racemosus</i>	2.0	–	–	–	2.0	1.0	2.60
<i>Nigrospora oryzae</i>	3.0	1.0	1.0	–	5.0	2.50	6.50
<i>Penicillium janthinellum</i>	2.0	1.0	2.0	–	5.0	2.50	6.50
<i>Penicillium sp.</i>	3.0	1.0	1.0	2.0	7.0	3.50	9.09
<i>Pestalotiopsis microspora</i>	2.0	1.0	–	–	3.0	1.50	3.90
<i>Phoma sp.</i>	–	1.0	–	–	1.0	0.50	1.30
<i>Trichoderma viridae</i>	3.0	–	1.0	–	4.0	2.0	5.19
<i>Trichothecium sp.</i>	1.0	–	–	–	1.0	0.50	1.43
Sterile mycelia	3.0	2.0	1.0	1.0	7.0	3.50	9.09
No. of isolates recovered	37.0	17.0	15.0	8.0	77.0	38.5%	

CF- colonization frequency of endophytes based on 200 bark segments plated, Db- Dibang valley; Tw- Tawang valley.

Fusarium avenaceum were isolated as endophytes from *Taxus* bark. This is similar to the finding of Rajagopal and Suryanarayanan [29] who also obtained *Fusarium avenaceum* as foliar endophytes of neem (*Azadirachta indica*). Amongst the isolated Endophytes, three species viz., *Cladosporium cladosporioides*, *Drechslera sp.* and *Phoma sp.* were isolated only from Tawang valley. Species like *Mucor racemosus*, *Acremonium acremonium*, *Trichothecium sp.*, *Trichoderma viride*, *Curvularia lunata* and *Fusarium moniliforme* were encountered only in Dibang valley. The percentage isolation of these location specific endophytes at both the locations was more during monsoon. The difference in occurrence of endophytes as regards to season and location, therefore, indicated the seasonality and location-specific nature of the endophyte assemblages. Such observation was also made, while studying endophytic fungal assemblages in inner bark of *Terminalia arjuna* collected during different seasons at different locations [5]. The two locations differed in species richness during the two seasons. During the monsoon, the samples obtained from Dibang valley harbored more isolates consequently had greater species richness. This may again be attributed to the favorable environmental conditions during monsoon that supported the colonization of endophytes better than the winter season. In Tawang valley although the number of fungi isolated during winter was less the species, diversity was high (Table 3). This may be due to the presence of dominant generalists and low frequency of occurrence of host specific forms. Diversity indices provide important information about rarity and commonness of species in a

community. The ability to quantify diversity in this way is an important tool for biologists trying to understand community structure. Therefore, different diversity indices were calculated. The Simpson and Shannon diversity indices differed between the two locations as well as the seasons. Endophytes recovered from bark samples collected during monsoon showed higher diversity index. This is identical with the finding of Tejesvi et al. [5] who, while studying endophytic fungal assemblages from inner bark and twig of *T. arjuna* found higher diversity index during monsoon. The two locations harbored 5 to 37 endophyte species and the similarity index was low during winter and high during monsoon (Table 4). The higher values of the both the indices during monsoon indicated higher species diversity in monsoon as compared to that of the winter season. This indicated that the isolates were more similar during the monsoon than they were during the winter season suggesting that similarity and rarity of species might have been controlled by the seasonal variations.

Endophytic fungi living asymptotically in plant tissues have also been recognized as a repository of novel secondary metabolites for potential therapeutic use. In this study we have demonstrated that crude extracts from the culture broth of endophytic fungi grown aerobically on Potato Dextrose broth medium displayed considerable antimicrobial activity against a panel of human pathogenic microorganisms. Out of 77 endophytic isolates, 15 strains (19.5%) showed antimicrobial activity inhibiting at least one of the test pathogens (Table 5). Among the potent strains 20% displayed antibacterial activity against

Table 3 Species richness and diversity of endophytic fungi associated with bark of *Taxus baccata*.

Season/location	Total isolate	Species richness	Diversity indices	
			Shannon	Simpson
Monsoon				
Dibang	37	17	2.767	0.959
Tawang	17	14	2.588	0.978
Winter				
Dibang	15	10	2.211	0.943
Tawang	08	05	1.557	0.893

Table 4 Number of total species and similarity co-efficient of endophytes with respect to different locations and seasons

Location/season	Species	Similarity co-efficient
Dibang		
Monsoon	37	0.39
Winter	15	0.20
Tawang		
Monsoon	17	0.56
Winter	08	0.34

both Gram-positive and Gram-negative bacteria inhibiting at least one-test organisms from the both while 40% exhibited both antibacterial and antifungal activity. Further, 46.7% displayed anti-candida activity. Out of the potent endophytic strains, metabolite produced by an endophytic fungus identified as *Fusarium sp.* displayed significant antimicrobial activity against the entire range of test pathogens. The compound exhibited strong antifungal and antibacterial activity. To assess the magnitude of antimicrobial action, the compound was co-assayed with reference antibiotic

Table 5 Antimicrobial activity of metabolites obtained from potent endophytic strains

Endophytic fungi	Zone of inhibition (mm)							
	Gram-negative bacteria			Gram-positive bacteria			Fungi	
	Se	Sa	Bs	Kp	Ec	Sf	Ca	Ct
<i>A.versicolor</i>	06.3 ± 1.5	0	0	04.6 ± 0.6	0	0	0	05.6 ± 1.1
<i>Colletotrichum</i> sp.	0	0	12.3 ± 0.5	0	0	0	08.6 ± 1.1	0
<i>P. janthinellum</i>	0	0	13.6 ± 1.5	0	0	0	0	0
<i>F.moliniforme</i>	0	14.6 ± 0.5	0	10.6 ± 1.1	0	0	0	0
<i>Mycelia sterilia</i> sp. 1	0	0	06.3 ± 0.6	0	0	0	0	0
<i>Mycelia sterilia</i> sp. 2	0	0	0	0	0	0	13.3 ± 1.5	08.3 ± 0.5
<i>Asperigillus</i> sp.	0	10.6 ± 1.1	05.6 ± 0.6	0	0	0	0	0
<i>Trichoderma viridae</i>	0	0		0	0	10.3 ± 1.5	0	0
<i>Fusarium</i> sp.	20.3 ± 0.5	18.6 ± 1.1	18.3 ± 1.5	27.0 ± 1.7	16.3 ± 0.5	24.3 ± 1.1	20.3 ± 0.5	10.6 ± 1.1
<i>A.alternata</i>	12.3 ± 2.5	0	0	0	0	0	0	0
<i>F.avenaceum</i>	0	0	0	06.3 ± 1.1	07.6 ± 2.5	0	10.6 ± 1.1	0
<i>Penicillium</i> sp.	0	11.3 ± 1.1	0	0	0	0	0	0
<i>Mycelia sterilia</i> sp.	3	0	12.0 ± 0	13.3 ± 1.5	0	0	0	08.6 ± 1.1 06.6 ± 1.1
<i>Curvularia lunata</i>	0	0	0	07.3 ± 0.6	0	0	0	0
<i>Trichothecium</i> sp.	08.0 ± 1.7	0	0	0	0	0	10.0 ± 0	0
Tetracycline		24.0 ± 0.5	22.0 ± 0	24.3 ± 0.5	30.0 ± 0	08.6 ± 1.1	25.0 ± 0	00
Fluconazole	0	0	0	0	0	0	0	0
Negative control	0	0	0	0	0	0	0	0

Tetracycline (30 mcg/disc) Co-assayed antibiotic for bacteria; Fluconazole (10 mcg/disc) Co-assayed antifungal agent for fungi; Negative control Dimethyl sulphoxide (solvent used to dissolve the crude metabolite) 100 µL/cup, 0: no antimicrobial activity; mean ± SD; n = 3.

Fig. 1 Antibacterial activity of the metabolite produced by *Fusarium* sp. (Fm) against *Klebsiella pneumoniae* (a) and *Staphylococcus epidermidis* (b), Co-assayed with Tetracycline (Tc-30 mcg/disc).

i.e. Tetracycline and a reference antifungal agent i.e. Fluconazole. Highest inhibition zone diameter was observed against *Klebsiella pneumoniae* that was almost equal to the

reference antibacterial agent (Fig. 1a) followed by *Staphylococcus epidermidis* (Fig. 1b). Similarly, the compound exhibited strong antifungal activity against *Candida albicans*

Fig. 2 Antifungal activity of the metabolite produced by *Fusarium* sp. (Fm) against *Candida tropicalis* (a) and *Candida albicans* (b), Co-assayed with Fuconazole (Fz-10 mcg/disc).

(Fig. 2b) and moderate activity against *Candida tropicalis* (Fig. 2a). These fungal pathogens, however, were resistant to the reference antifungal agent, Fuconazole.

Endophytes are gaining importance because of their enormous potential to produce novel bioactive compounds. Many new and interesting bioactive metabolites such as antibiotics, antiviral, anticancer and antioxidants compounds, which are of pharmaceutical, industrial and agricultural importance have been reported and characterized from fungal endophytes [10]. This suggest the great potentiality of fungal endophytes as a sources of important bioactive molecules considering health problems like cancer, multi drug resistance, immergence of new diseases etc. the world is facing presently with. Fungal endophytes of *Taxus* sp. around the world have been extensively investigated for the production of Taxol [16, 18]. However, studies with regards to fungal diversity and their antimicrobial evaluation from *T. baccata* were still lacking. The present investigation is the first report on endophytic fungal diversity and antimicrobial potentiality of medicinally most important plant *Taxus baccata* from the Eastern-Himalayan global biodiversity hotspot. We are currently pursuing purification and characterization of metabolites to facilitate screening against therapeutic targets.

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