Ann Microbiol (2014) 64:721–734
DOI 10.1007/s13213-013-0707-9

ORIGINAL ARTICLE

Impact of environmental variables on the isolation, diversity and antibacterial activity of endophytic fungal communities from *Madhuca indica* Gmel. at different locations in India

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Received: 31 January 2013 / Accepted: 1 August 2013 / Published online: 28 August 2013
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Abstract A total of 1,897 isolates of endophytic fungi from *Madhuca indica* Gmel., representing 40 morphologically distinct fungal taxa were obtained from 2,700 segments of stem, bark and leaf from three different locations (Loc 1, Loc 2 and Loc 3) in Uttar Pradesh, India. Out of 40 taxa, 28 were identified microscopically and the remaining 12 by molecular methods. Coelomycetes (62.41 %) were the most prevalent fungal group followed by hyphomycetes (28.89 %) and ascomycetes (8.70 %). Colonisation frequency (CF) was greater in stem (82.55 %) than in leaf (65.00 %) and bark (63.22 %). Due to the dominance of a few taxa, species richness and Shannon and Simpson diversity indices were lower in stem than in leaf and bark at each location. Interestingly, less rainfall and lower temperatures disfavoured the overall colonisation of fungal endophytes at Loc 2. The stem samples from all locations were very similar in their endophytic composition, whereas bark and leaf samples showed differences. The dominant endophytic fungi isolated were *Phomopsis* sp. 1 (9.185 %), and *Colletotrichum gloeosporioides* (7.00 %). Principal component analysis showed 55 % tissue specificity with 51.08 % maximum variance. Antibacterial activity revealed that 58.33 % endophytic fungi were active against at least one or more bacterial pathogens, whereas the crude extract of five endophytic fungi inhibited the growth of five or more than five (50 %) of the pathogens tested. This report illustrates the value of having an adequate sample size from different tissues and different locations for species and chemical diversity in search of novel natural products.

Keywords Antimicrobial · GenBank · Mycoflora · Phylogeny · Secondary metabolites · Symbiosis

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Introduction

Fungal endophytes are recognised by their ability to live for part or all of their lifecycle internally and asymptomatically within plants (Wilson 1995). Fungal endophytes comprise a wide range of organisms believed to have evolved from a weakly pathogenic group of fungi that may have lost their virulence over time and have developed either a symbiotic relationship with their host or grow as latent pathogens (Saikkonen et al. 1998). On the basis of phylogeny and life history, endophytic fungi are grouped with the Clavicipitaceae family of fungi, which specifically colonise some restricted grasses and usually belong to the genera *Epichloe* and *Neotyphodium*. Non-clavicipitaceous fungi are highly diverse, with poorly defined ecological roles that occur in almost all plant species and usually belong to the ascomycotina (Rodriguez et al. 2009). Unlike mycorrhizal fungi, which colonise plant roots and are limited to the rhizosphere, endophytic fungi may grow in any host tissues, i.e. root, stem, twig, bark and leaf (Carroll 1988; Stone et al. 2004; Verma et al. 2007, 2011).

Out of a total estimated 1.5 million fungal species, only 6–7 % have yet been discovered (Hawksworth 2001). The remaining undiscovered fungi may be in the form of hidden endophytes, which gives us great hope for discovering new

taxa with potential bioactivity for new natural products. Endophytic mycoflora are reported from every group of plants, algae (Hawksworth 1987), bryophytes (Schulz et al. 1993), pteridophytes (Sati and Belwal 2005), gymnosperms (Carroll and Carroll 1978) and monocotyledonous (Clay 1988; Rodrigues 1994) and dicotyledonous (Lodge et al. 1996; Rajagopal and Suryanarayanan 2000; Verma et al. 2007; Gond et al. 2007; Kharwar et al. 2010, 2011b) angiosperms. They are also reported from mangroves (Suryanarayanan and Kumaresan 2000) and the complete parasitic plant *Cuscuta reflexa* (Suryanarayanan et al. 2005). Host plants provide a vibrant environment and habitat in which many factors influence the structure and composition of species that colonise different tissues. The community structure of endophytic fungi varies significantly both spatially and temporally in many higher plants (Arnold et al. 2000). Not much extensive work has been done on the structural and functional diversity of endophytic fungi of plants from the Indian subcontinent, but recent research using diversity analysis has begun searching for biologically active compounds from endophytes that may answer many problems of humanity in today's world (Kharwar and Strobel 2011). Endophytic fungi play a significant role in plant ecosystems by providing resistance against disease and insects, and countering abiotic stresses such as drought and the presence of metals (Rodriguez et al. 2009; Redman et al. 2002). Endophytic fungi now represent an established microbial group for the production of bioactive compounds with various biological functions, e.g. antimicrobial, anticancer, anti diabetic, antioxidant, immunosuppressive, antiviral, anti-inflammatory, antimalarial and insecticidal activities (Verma et al. 2009). Antimicrobial agents produced by endophytic fungi may provide new alternatives to combat multidrug-resistant human pathogens, the prevalence of which is increasing almost daily. They are also well known for producing enzymes of industrial importance. Taxol, a potent anti-breast cancer drug first discovered from the bark of *Taxus brevefolia*, is also produced by its endophytic fungus *Taxomyces andreanae* (Stierle et al. 1993), possibly due to horizontal gene transfer from host to endophyte. Similarly, other recent examples also point towards the potential to harvest active compounds such as vincristine, vinblastine, piperine and azadirachtin from the endophytic fungi of their original hosts (Zhang et al. 2000; Verma et al. 2011; Kusari et al. 2012). The diverse niches of their host plants may induce endophytes to produce a variety of secondary metabolites of significant use (Strobel and Daisy 2003).

Endophytic fungi have greater potential to produce novel and bioactive compounds than soil fungi. A literature search revealed that 80 % of endophytic fungi exhibited biological activity compared to 64 % of soil isolates, while more than 58 % of novel bioactive compounds have been isolated from endophytic fungi compared to only 38 % from soil fungi (Schulz et al. 2002). A recent review reported that more than 100 anticancer compounds have been recovered from endophytic fungi to date (Kharwar et al. 2011a). However, to fully exploit fungal endophytes for useful products, optimization of fermentation for production of the desired compound, clinical tests, and the approval of the government/medical board of the relevant country are essential. Nevertheless, these initial steps in natural product discovery bode well for future endeavours in the screening of native plants for endophytes, and their systematic study in order to isolate and characterise biologically active products.

Madhuca indica Gmel., synonym *Madhuca longifolia* (L.) J.F. Macbr. var. *latifolia* (Roxb.) A. Chev., also known as butter tree, is indigenous to central India and is the main constituent of forest vegetation. It is a highly medicinal plant and its importance for the treatment of various diseases and disorders, like rheumatism, diabetes, skin diseases, piles and chronic constipation, has been described in Ayurveda. *Madhuca* seeds contain a high amount of fat, and can be used successfully for the production of washing soaps. Its flowers are eaten as vegetables, and used for making vinegar and distilled liquor, which is used to treat coughs and skin diseases. Terpenoides, saponins (Yoshikawa et al. 2000) along with madhucosides A and B, and protobasic acid glycosides, which inhibit the release of free radicals for phagocytes, have been reported from this plant (Pawar and Bhutani 2004). Chloroform, acetone and ethanolic extracts of leaf, stem and barks have displayed antibacterial activity (Khond et al. 2009). The methanolic extract of bark of *M. indica* has significant potential to decrease the elevated levels of blood glucose, cholesterol, triglycerides, and high density lipoprotein (HDL) in diabetic rats (Kumar et al. 2011).

Considering the ethnobotanical history, medicinal importance and indigenous distribution of *M. indica*, we selected this plant for the present study with a view to exploring its endophytic fungal diversity with morphological and molecular identification and further to screen for antibacterial potential. There are only a few reports on endophytic fungi from the Indian subcontinent on different hosts such as *Azadirachta indica*, *Terminalia arjuna*, *Adhatoda zeylanica*, *Bauhinia phoenicea*, *Callicarpa tomentosa*, *Clerodendron serratum*, *Labelia nicotinifolia*, *Crataeva magna*, *Aegle marmelos*, *Eucalyptus citriodora* and *Tinospora cordifolia* (Nalini et al. 2005; Raviraja 2005; Tejesvi et al. 2005; Gond et al. 2007; Verma et al. 2007; Kharwar et al. 2010; Mishra et al. 2012; Chowdhary et al. 2013). To date, only 12 endophytic fungi have been reported from leaves of *M. longifolia* L. (Synonym of *M. indica*) (Thalavaipandian et al. 2011); interestingly, these have antimicrobial activity. To the best of our

knowledge, other than that of Thalavaipandian et al. (2011), there is no other literature report, at least with adequate sampling, on the endophytic fungal diversity and antibacterial activity of *M. indica*.

Materials and methods

Sampling

Samples were collected randomly from three different locations (Loc 1–3): the campus of Banaras Hindu University (BHU) in Varanasi district (Loc 1; relatively moist and less polluted area), agriculture lands of Chandauli district, U.P. India (Loc 2), and the Hathinala forest of Sonebhadra district (Loc 3; natural forest), from October 2009 to September 2010. Sampling was done in triplicate for each plant part (stem, leaf and bark). A total of 27 samples were collected (9 from each location and 3 from each tissue) on each occasion. All samples were brought to the laboratory in an icebox, stored at 4 °C in refrigerator and were used within 48 h of collection. The location's characteristics were noted.

Surface sterilisation, isolation and identification

All samples were washed thoroughly under running tap water followed by double distilled water before processing. To eliminate epiphytic microorganisms, all samples were initially surface sterilised using standard methodology (Petrini et al. 1992). The samples were immersed in 75 % ethanol for 2–3 min, then sterilised with aqueous sodium hypochlorite (4 % available chlorine) for 2–5 min before rinsing in 75 % ethanol for 30 s. The tissues were then rinsed three times in sterile double distilled water and allowed to surface dry under sterile conditions. The samples were cut into small pieces (stems into 0.25 cm thick sections, leaves into 0.5×0.5 cm squares, and bark into 0.5×0.5 cm squares) using a sterile cutter. A total of 2,700 segments were plotted from 27 samples.

Four to six segments of plant tissue were placed on each potato dextrose agar [PDA; containing streptomycin (150 μg/ml)] Petri plate, and sealed with Parafilm™ to avoid air contamination. Petri plates were incubated in a BOD incubator (Calton super deluxe, Narang Scientific Works, New Delhi) for 21 days on a 12-h light/dark cycle at 26±2 °C. The plates were observed regularly on alternate days and hyphal tips of actively growing fungi were transferred to fresh PDA plates. Endophytic fungal genera and species were identified manually, according to their macroscopic and microscopic characteristics such as fruiting structures and spores morphology, using standard taxonomic manuals (Barnett and Hunter 1998; Von Arx 1978; Ellis 1976; Ainsworth et al. 1973). Molecular methods were used to characterise unidentified/or non-sporulated fungal isolates. All isolated and identified fungi were maintained in cryovials layered with 15 % glycerol and also in a lyophilised condition at −20 °C in a deep freezer (Blue Star). Isolates were deposited with the Department of Botany, BHU, India under their numbers MMTL MIRNK01–1897.

Molecular characterization of unidentified endophytic fungal isolates

Genomic DNA was extracted from unidentified isolates of endophytic fungi following the slightly modified protocol of Sim et al. (2010). The universal primers ITS-1: 5′TCCGTAG GTGAACCTGCGG 3′ and ITS-4: 5′TCCTCCGC TTATTGATATGC3′ (GeNei) (White et al. 1990) were used to amplify the 5.8S rDNA and two ITS regions between the 18S and 28S rDNA. Total PCR mixtures of 25 μl, each contained 1 μl (100 ng/μl) DNA template, 1 μl each primer, 0.33 μl (3 units/μl) *Taq* polymerase, 1.5 μl of 25mM $MgCl_2$, 0.25 μl dNTPs, buffer (10X) 2.5 μl and 17.42 μl MQ water for each reaction mixture. PCR reactions were performed in My-cycler (BioRad, Hercules, CA) under the following conditions: pre-denaturation at 94 °C for 4 min, 35 cycles at 94 °C (denaturation) for 1 min, 55 °C (annealing) for 1 min, 72 °C (extension) for 1 min and then a final extension for 5 min at 72 °C. Amplified PCR products were resolved by electrophoresis in a 1.5 % (w/v) agarose gel stained with ethidium bromide (0.5 μg/ml) for visual examination. PCR products were sent to Chromas Biotech (Sahakara Nagar, Bangalore, India) for sequencing. The ITS rDNA sequences obtained were used to retrieve similar sequences using the NCBI BLAST program from the NCBI GenBank sequence database. MEGA version-5 software was used to align the sequences and for phylogenetic tree construction. The phylogenetic tree was evaluated by performing bootstrap analysis with evolutionary distances using the neighbour joining (NJ) method (Saitou and Nei 1987). The rDNA sequences have been submitted to NCBI GenBank database (with accession numbers).

Statistical analysis of data

The colonisation frequency (%CF) of endophytic fungi was calculated using the formula given by Hata and Futai (1995). $\%CF = N_{col} / N_t \times 100$, where N_{col}=number of segments colonized by each fungus, and N_t=total number of segments studied. Simpson's, Shannon–Wiener diversity indices and species richness were calculated by PAST software (http://folk.uio.no/ohammer/past/) using the following formula;

Simpson's Diversity=$1-\sum (pi)^2$, Shannon–Wiener diversity=$-\sum s$ (pi log pi), where pi=proportion of frequency of the ith species in a sample. Species evenness was calculated as; Evenness (E)=$H / \log(S)$. Where H=Shannon Wiener diversity and S=species richness, i.e. total number of species. ANOVA was performed using SPSS 16.0 software to detect significant differences among mean diversity indices of the entire sample collected from different location and tissues. For ecological association, principal component analysis (PCA) was performed using PAST software to determine the interrelationship and specificity of endophytic mycoflora among different tissue samples and sites (Orduña et al. 2011).

Antibacterial assay

Twenty four endophytic fungi were screened for antibacterial activity against ten potential human pathogenic bacteria: *Enterococcus faecalis* (IMS/GN7), *Escherichia coli* (ATCC 25922), *Salmonella typhi* (MTCC 3216), *Pseudomonas aeruginosa* (ATCC27853) *Shigella boydii* (IMS/GN2), *Salmonella enteritidis* (IMS/GN3), *Morganella morganii* (IMS/GN6), *Staphylococcus aureus* (ATCC 25923), *Aeromonas hydrophila*, (IMS/GN11) and *Proteus mirabilis* (IMS/GN13) (all procured from the Institutes of Medical Sciences (IMS), BHU, Varanasi). Endophytic fungal isolates were cultivated in 500 ml Erlenmeyer flasks containing 250 ml potato dextrose broth (PDB) and incubated for 21 days at 26± 2 °C under static conditions in a biochemical oxygen demand (BOD) incubator. The broth of each flask was filtered through Whatman # 1 filter paper, and extracted three times with 150 ml ethyl acetate.

The pooled ethyl acetate extract was then evaporated to dryness in a vacuum rotary evaporator (IKA RV 10, IKA, Staufen, Germany) and weighed. Filter paper discs (5 mm diameter) were impregnated with 10 µl crude extract (0.5 mg/µl in methanol) and placed on the surface of Mueller-Hinton medium already seeded with the test bacterium in a Petri plate. One negative control disc impregnated with 10 µl methanol only was also set up for each test bacterium. Discs loaded with 5 µg ciprofloxacin were placed for each test bacterium as a positive control. The plates were incubated at 37±2 °C for 24 h and measured inhibition zones. Each test was done in triplicate and interpretations were made on the average values of results.

Results

Phylogeny

A total of 1,897 endophytic fungal isolates were recovered from the asymptomatic stem, bark and leaf tissues of *Madhuca indica* from three different locations. Most (1,606) of these isolates belong to 28 morphologically identified distinct taxa, while the remaining 291 unidentified isolates were categorized into another 12 distinct taxa and identified by molecular methods. A single isolate of each unidentified morphotype was taken for molecular identification and phylogenetic analysis. The GenBank accession numbers of all 12 sequences with their percentage similarity with the closest species is given in Table 1. The phylogenetic tree constructed was rooted with MIS 01 isolates, and the 12 morphotypes fall into nine main groups (Fig. 1). In the first group, isolates MIB06 and MIB03 showed 100 % bootstrap values with *Aspergillus* sp., MIB02 showed 100 % bootstrap values with *Emericella variecolor* and grouped with *Emericella* spp., isolate MIB01 showed 100 % bootstrap values with *Botryosphaeria* and *Lasidiodiplodia* sp., MIB08 and MIB 04 isolates showed 99 % and 93 % bootstrap values with

Table 1 GenBank accession numbers and their % similarity with closest taxa

Isolate	Accession no.	Similarity (%)	Closest species	Accession no. of closest species
MIS01	JN163854	98 %	*Fusicoccum* sp. (NR-2006-D13)	DQ480351.1
MIS02	JN163855	94 %	*Phomopsis liquidambri* (MJ08)	HQ328002.1
MIS03	JN163856	97 %	*Phomopsis* sp. (H1)	AB286211.1
MIL01	JN163853	97 %	*Preussia* sp.	HQ607945.1
MIB01	JN030354	96 %	*Botryosphaeria rhodina* (SY5M17)	JF923831.1
MIB02	JN030355	92 %	*Emeriecella* sp. (NRRL212).	EF652435.1
MIB03	JN030356	99 %	*Aspergillus lentulus*(NRRL35553)	EF669970.1
MIB04	JN030357	98 %	*Pleospora* sp. (VegaE2-15)	EU002891.1
MIB05	JN030358	97 %	*Fusarium* sp. (1-66)	DQ780423.1
MIB06	JN163852	99 %	*Aspergillus tubingensis* (YMCA)	JF436888.1
MIB07	JN604095	97 %	*Xylariales* sp. (VegaE4-79)	EU009996.1
MIB08	JN604096	99 %	*Pleospora* sp. (E9902a)	JN637946.1

Pleospora spp., and grouped with *Pleospora* spp. MIB 05 showed 96 % bootstrap value with *Fusarium* sp. MIS 02 and MIS03 showed 90 % bootstrap value with *Phomopsis* sp. and MIL01 showed 100 % bootstrap value with *Preussia* sp. and grouped in to *Preussia* spp. MIB07 showed 91 % bootstrap value with *Xylaria* sp.

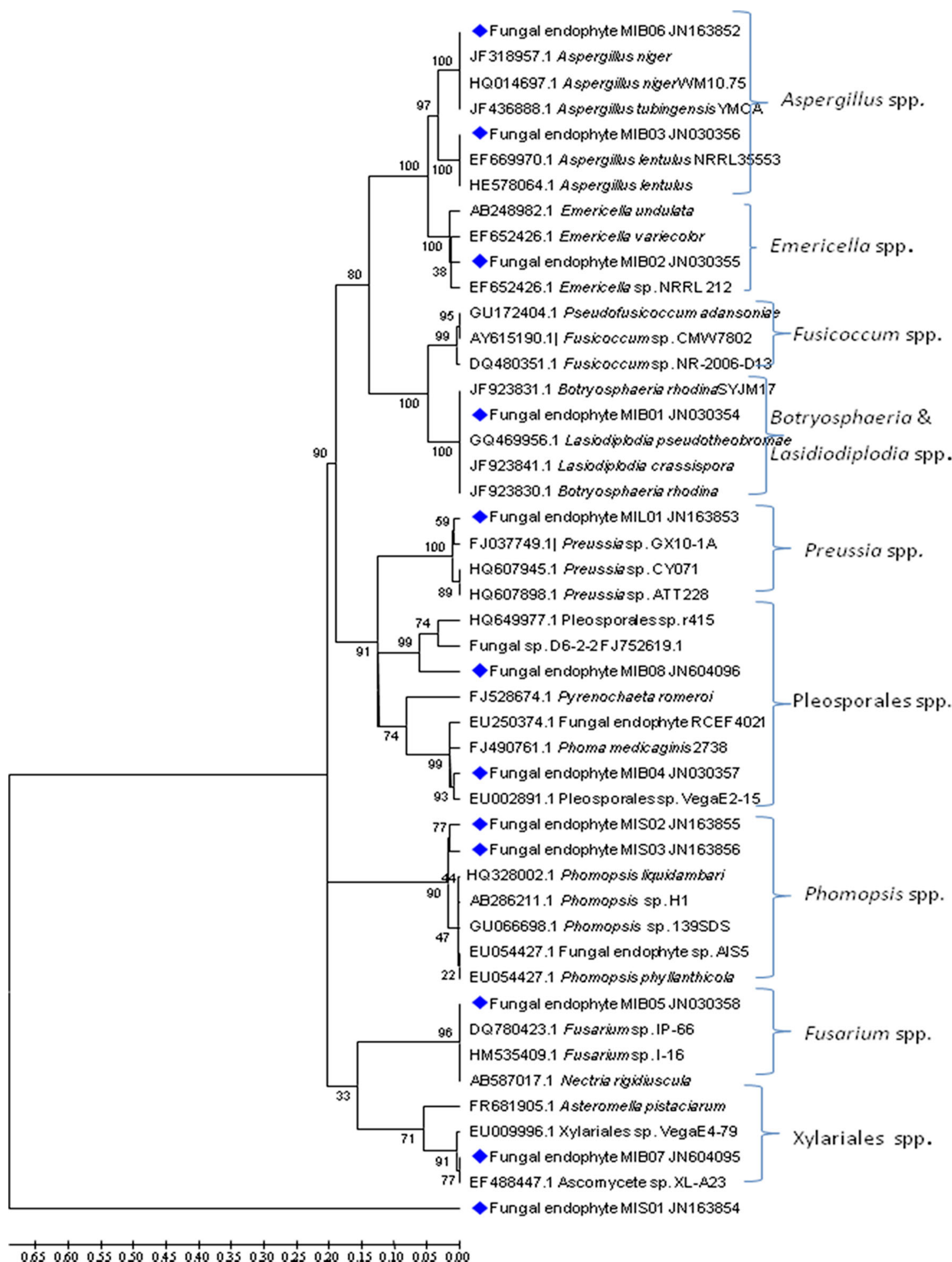

Fig. 1 Phylogenetic relationship of molecularly identified fungal endophytes from *Madhuca indica* with some recovered sequences from the NCBI GenBank data base. The tree was constructed and evaluated by performing bootstrap analysis with the evolutionary distances using the neighbour joining method

Table 2 Occurrence of endophytic fungi in stem, bark and leaf tissues of *Madhuca indica* at three diffrent locations. *BHU* (Banaras Hindu University)

Endophytic fungus	BHU			Chandauli			Hathinala			Total	%CF	% Dominance
	Stem	Bark	Leaf	Stem	Bark	Leaf	Stem	Bark	Leaf			
Hyphomycetes												
Alternaria alternata	0	5	14	0	0	10	11	0	5	45	1.6667	2.37217
Alternaria sp.	0	0	6	0	9	14	0	8	26	63	2.3333	3.32103
Aspergillus flavus	0	4	7	6	0	7	0	9	0	33	1.2222	1.73959
A. fumigatus	0	5	5	0	0	0	0	7	0	17	0.6296	0.89615
A. niger	4	6	7	0	2	0	0	0	0	19	0.7037	1.00158
A. terreus	0	6	0	0	0	0	0	0	0	6	0.2222	0.31629
Aspergillus sp.	0	0	0	0	0	4	0	0	0	4	0.1481	0.21086
Cladosporium cladosporioides	0	17	9	0	12	7	0	8	0	53	1.963	2.79389
Cladosporium sp.	0	2	0	0	0	0	0	3	0	5	0.1852	0.26357
Corynespora sp.	0	0	0	0	0	6	0	0	28	34	1.2593	1.7923
Fusarium graminearum	0	0	0	0	0	0	0	8	0	8	0.2963	0.42172
Fusarium sp.	8	10	13	11	13	0	15	8	8	86	3.1852	4.53347
Monodictys sp.	0	0	15	0	0	0	0	0	0	15	0.5556	0.79072
Penicillium sp. 1	0	6	6	0	3	0	0	18	7	40	1.4815	2.10859
Penicillium sp. 2	0	0	0	0	0	0	0	4	0	4	0.1481	0.21086
Gliocladium sp.	0	0	0	0	0	0	0	0	4	4	0.1481	0.21086
Taeniolella sp.	0	3	0	0	0	0	0	0	0	3	0.1111	0.15814
Trichoderma sp.	0	9	0	6	4	0	0	20	6	45	1.6667	2.37217
Aspergillus tubingensis MIB 06[a]	0	0	0	0	0	0	0	2	0	2	0.0741	0.10543
Aspergillus lentulus MIB03[a]	0	0	0	0	0	0	0	7	0	7	0.2593	0.369
Fusarium sp. MIB05[a]	0	23	0	0	32	0	0	0	0	55	2.037	2.89931
Coelomycetes												
Aschersonia sp.	33	0	8	33	4	0	24	0	0	102	3.7778	5.37691
Botryodiplodia sp.	0	0	0	0	0	0	6	0	0	6	0.2222	0.31629
Colletotrichum coccodes	35	4	35	13	0	39	0	0	10	136	5.037	7.16921
C. gleosporioides	51	0	0	36	0	20	50	0	32	189	7	9.9631
Macrophoma sp.	0	0	17	0	0	52	0	0	74	143	5.2963	7.53822
Pestalotia sp.	0	0	8	0	34	0	0	69	12	123	4.5556	6.48392
Phlyctaena sp.	0	30	0	0	0	0	0	0	0	30	1.1111	1.58144
Phomopsis sp. 1	70	0	0	70	0	0	90	0	18	248	9.1852	13.0733
Phomopsis sp. 2	36	0	0	46	0	0	44	0	11	137	5.0741	7.22193
Phomopsis liquidambari MIS02[a]	0	0	0	12	0	0	0	0	0	12	0.4444	0.63258
Phomopsis sp. MIS03[a]	0	0	0	16	0	0	0	0	0	16	0.5926	0.84344
Botryosphaeria rhodina MIB01[a]	0	19	0	0	67	0	11	26	21	144	5.3333	7.59093
Ascomycetes												
Chaetomium sp.	0	0	8	0	0	0	0	0	0	8	0.2963	0.42172
Pleospora sp. MIB04[a]	0	12	0	0	0	0	0	0	0	12	0.4444	0.63258
Preussia sp. MIL01[a]	0	0	6	0	0	0	0	0	0	6	0.2222	0.31629
Fusicoccum sp. MIS07[a]	6	0	0	0	0	0	0	0	0	6	0.2222	0.31629
Emericella sp. MIB02[a]	0	0	0	0	0	0	0	4	0	4	0.1481	0.21086
Xylaria sp. MIB07[a]	0	14	0	0	0	0	0	0	0	14	0.5185	0.73801
Pleospora sp. MIB08[a]	0	13	0	0	0	0	0	0	0	13	0.4815	0.68529
Total	243	188	164	249	180	159	251	201	262	1,897	70.26	100

[a] Identified by molecular methods

Table 3 Colonisation frequency (% CF) of three tissues of *M. indica* from three diffrent locations

Plant part	Location			
	BHU	Chandauli	Hathinala	Total
Stem	81.00	83.00	83.667	82.556
Bark	62.667	60.00	67.00	63.22
Leaf	54.667	53.00	87.333	65.00
Total	66.111	65.333	79.333	70.259

Fungal diversity

A total of 1,897 isolates was recovered from 2,700 segments of three different tissues samples of *M. indica* collected at three different locations (see Materials and methods) and grouped into 40 morphologically distinct fungal species. Maximum %CF was found with the fungus *Phomopsis* sp. 1 (9.18 %) followed by *Colletotrichum gloeosporioides* (7.00 %), *Macrophoma* sp. (5.29 %), *Botryosphaeria rhodina* (5.33 %), *Phomopsis* sp. 2 (5.07 %), and *Colletotrichum coccodes* (5.03 %); interestingly, these all belong to coelomycetes. In the hyphomycetes, *Fusarium* sp. had maximum %CF (3.18 %) (Table 2). The total colonisation of endophytic fungi was highest in all three tissues collected from Hathinala followed by BHU and then Chandauli (Table 3). Of the three tissues, maximum colonisation was found in stem followed by leaf and bark. Stem showed maximum %CF from locations Hathinala and Chandauli and minimum %CF at BHU (Table 3).

Unlike previous reports, most isolates recovered were coelomycetes (62.41 %) followed by hyphomycetes (28.89 %) with the fewest being ascomycetess (8.70 %) (Fig. 2). Coelomycetes isolates were recovered mostly from stem samples at all three locations, i.e. BHU (Loc 1), Chandauli (Loc 2), and Hathinala (Loc 3), with 10.12 %, 10.17 % and 9.38 %, followed by leaf tissue (3.16 %, 5.85 % and 10.59 %), and bark tissue (2.79 %, 5.32 % and 5.007 %) (Fig. 3). Most hyphomycetes isolates were recovered from bark at each location (Loc 1, 2, 3: 5.06 %, 3.95 % and 5.37 %, respectively) followed by leaves (4.32 %, 2.53 % and 1.37 %) and least from stems (0.6 %, 1.21 % and 4.42 %). Ascomycetes were located mainly in stems from BHU (2.055 %) and Chandauli (1.74 %) followed by bark at BHU, Chandauli and Hathinala (2.055 %, 0.21 % and 0.21 %), and were lowest in leaf tissue from BHU and Hathinala (1.15 % and 1.26 %), respectively (Fig. 3). No ascomycetes were recovered from stems at Hathinala or from leaves at Chandauli (Fig. 3). The abundance of endophytic isolates was greatest for coelomycetes (1,286) followed by hyphomycetes (548) and ascomycetes (63), while Shannon-Weiner and Simpson diversity indices were greater in hyphomycetes, followed by coelomycetes and ascomycetes (Table 4). Dominance showed an inverse relationship with diversity indices, i.e. maximum to ascomycetes (0.166) followed by coelomycetes (0.1225) and least to hyphomycetes (0.087).

Diversity indices of all three tissues, separately and in total, were calculated at each location. Overall endophytic fungal diversity indices determined were Shannon index 3.049, Simpson 0.936, evenness 0.527 and dominance 0.0637

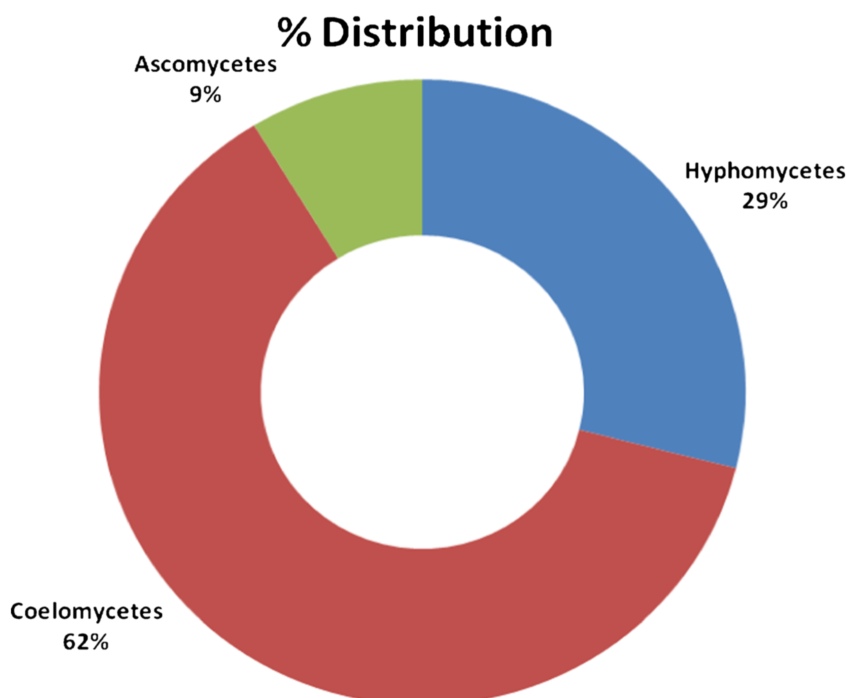

Fig. 2 Percentage distribution of endophytic fungi isolated from *M. indica* in three different fungal groups

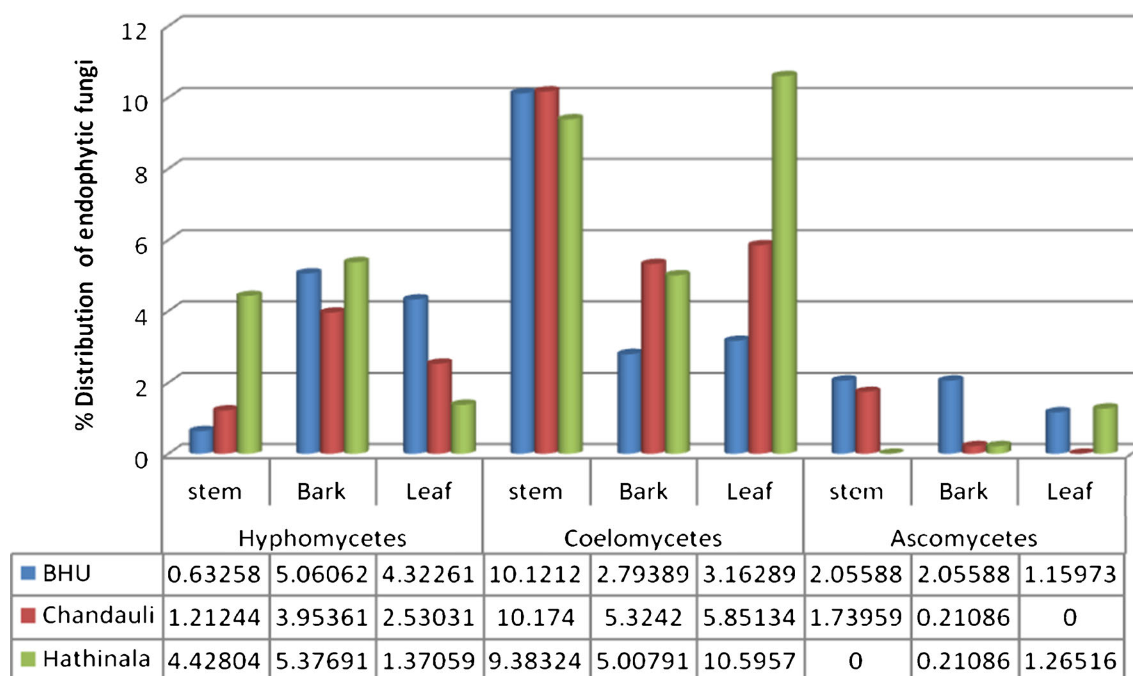

Fig. 3 Distribution (%) of endophytic fungi of *M. indica* in three different tissues (stem twig, bark and leaf) at three different locations. *BHU* (Banaras Hindu University)

(Table 5). In comparison, among the three different locations, maximum Shannon and Simpson indices were found in samples from Loc 1 (3.041, 0.938) followed by Loc 2 (2.746 and 0.925) and Loc 3 (2.736 and 0.916). The diversity pattern of endophytic fungi with respect to different tissues was not same at all three locations. The bark tissue at Loc1 had maximum Shannon and Simpson indices (2.655 and 0.915) followed by leaf (2.534 and 0.903), with minimum indices in stem (1.79 and 0.809). At Loc 3, maximum Shannon and Simpson indices were found in leaf tissue (2.29 and 0.864) followed by Bark (2.228 and 0.8358), with minimum values in stem (1.75 and 0.78), while at Loc 2, stem had maximum Shannon and Simpson indices (2.01 and 0.836) followed by leaf (1.85 and 0.794), with a minimum in bark (1.797 and 0.7806) (Table 5). Shannon indices obtained at different locations from various tissues were used for analysis of variance (ANOVA), which showed that all three tissues (stem, leaf and bark) at each location and all three locations (Loc 1,2,3) from each tissue varied significantly ($P \leq 0.01$).

Table 4 Diversity of fungal endophytes to different fungal groups

Index	Hyphomycetes	Coelomycetes	Ascomycetes
Richness	21	12	7
Individuals	548	1,286	63
Dominance_D	0.08787	0.1225	0.1665
Shannon_H	2.624	2.201	1.861
Simpson_1-D	0.9121	0.8775	0.8335
Evenness	0.6567	0.753	0.9183

Ecological association

Principal component analysis revealed two components or axes (1, 2), with maximum 51.08 % variation and an Eigenvalue of 764.65 (Fig. 4). PCA exposed the tissue specific affinity of some endophytic fungal isolates. For instance, 13 taxa [*Aspergillus terreus*, *A. tubingensis*, *A. lentulus*, *Fusarium* sp., *Cladosporium* sp., *Fusarium graminearum*, *Penicillium* sp. 2, *Taeniolella* sp., *Phlyctaena* sp., *Pleospora* sp. (MIB08), *Emericella* sp., *Xylaria* sp. and *Pleospora* sp. (MIB04)] were isolated only from bark, and 7 taxa (*Aspergillus* sp., *Gliocladium* sp. *Macrophoma* sp., *Chaetomium* sp., *Preussia* sp., *Corynespora* sp. and *Monodictys* sp.) only from leaf, whereas 4 taxa (*Botryodiplodia* sp., *Phomopsis liquidambari*, *Phomopsis* sp. and *Fusicoccum* sp.) were confined to stem only. Of 40 endophytic fungal taxa obtained, 24 (60 %) showed restricted association with a specific tissue, whereas 16 showed association with more than one tissue. Eight endophytic fungal taxa, i.e. *Alternaria alternata*, *Aspergillus flavus*, *A. niger*, *Fusarium* sp., *Trichoderma* sp., *Aschersonia* sp., *Colletotrichum coccodes* and *Botryosphaeria rhodina* were found to be associated with all tissues and thus, they were not specific to any tissue (Table 1).

Antibacterial activity

Out of 24 endophytic fungi screened, 14 (58.33 %) inhibited the growth of at least one or more of the bacterial pathogens tested (Table 6). Five endophytic fungi [*Aschersonia* sp.

Table 5 Diversity indices of endophytic fungi recovered from three different tissues, i.e. stem, bark and leaf of *M. indica* at three diffrent locations

Index		BHU (Loc 1)	Chandauli (Loc 2)	Hathinala (Loc 3)	Total
Species richness	Stem	8	10	8	15
	Bark	18	10	15	26
	Leaf	15	9	14	23
	Total	29	21	25	40
Shannon_H	Stem	1.791	2.01	1.751	2.007
	Bark	2.655	1.797	2.228	2.68
	Leaf	2.534	1.85	2.297	2.628
	Total	3.041	2.746	2.736	3.049
Simpson_1-D	Stem	0.8099	0.8361	0.7839	0.819
	Bark	0.9159	0.7806	0.8356	0.8991
	Leaf	0.903	0.7994	0.8648	0.8913
	Total	0.9385	0.9251	0.9169	0.9363
Evenness	Stem	0.7492	0.7465	0.7198	0.4962
	Bark	0.7904	0.6063	0.6187	0.561
	Leaf	0.8406	0.7068	0.71	0.602
	Total	0.7215	0.7419	0.6169	0.5272
Dominance_D	Stem	0.1901	0.1639	0.2161	0.181
	Bark	0.08409	0.2194	0.1644	0.1009
	Leaf	0.09697	0.2006	0.1352	0.1087
	Total	0.06154	0.07491	0.08308	0.06375

(MIL13), *Aspergillus* sp. (MIL04), *Botryosphaeria rhodina* (MIB01), *Pleospora* sp. (MIB04) and *Fusarium* sp. (MIB05)] inhibited the growth of five or more than five (50 %) bacterial pathogens. Two endophytic fungi [*Aschersonia* sp. (MIL13) and *Aspergillus* sp. (MIL04)] inhibited more than 70 % of tested human bacterial pathogens including both Gram positive and Gram negative species, which confirm their broad spectrum of activity. The pathogenic bacteria most sensitive to the crude extract of the tested endophytic fungi were *Shigella boydii*, *Salmonella typhi* and *Staphylococcus aureus*, which were inhibited by more than 7 endophytic fungi out of the 24 tested. *Aschersonia* sp. (MIL13) inhibited the growth of nine out of ten the tested bacterial pathogens, with 7 bacterial types showing an inhibition zone of more than 13 mm. Crude extract of *Botryosphaeria rhodina* exhibited maximum inhibition zone against *Shigella boydii* (25.33±1.00), whereas *Aspergillus* sp. (MIL04) showed maximum activity against *S. aureus* (19.00±1.00 mm). *Proteus mirabilis* was the most resistant bacterium, being inhibited only mildly by only 3 endophytic fungi out of 24.

Discussion

The presence of endophytic fungi in every plant yet studied is well documented, but the number of plants investigated for this purpose is very low. The benefits provided by endophytic fungi to plants against different stresses are well known; however, proper identification, documentation and bioassay are the preliminary steps necessary to exploit the potential of these relatively untapped endophytic microbes.

Microscopic analysis of identifiable endophytic fungi using standard manuals is a traditional, and reliable, approach still used by many researchers (Verma et al. 2007; Kumar and Hyde 2004; Gond et al. 2007, 2012; Kharwar et al. 2011b). However, molecular approaches are now being applied to the identification of significant numbers of mycelia sterilia and morphologically closely related species isolated as endophytic fungi, or to those unable to produce identifiable spores (Zhang et al. 1997). Recently, several molecular techniques, such as DNA cloning (Seena et al. 2008), DGGE (Duong et al. 2006),

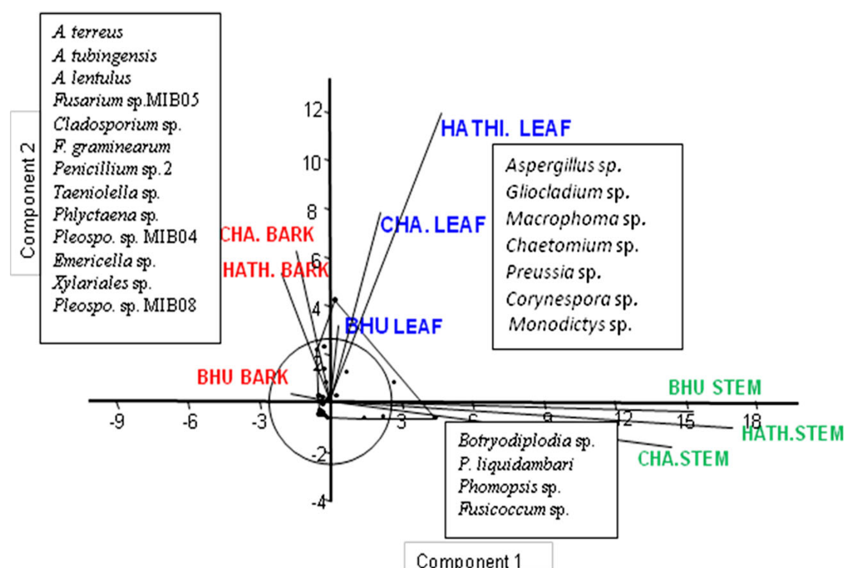

Fig. 4 Principle component analysis (PCA) of fungal endophytes recovered from stem, bark and leaf tissue of *M. indica* revealed 51.08 % of total variation with an Eigenvalue of 764.65

Table 6 Antibacterial activity of crude extracts (5 mg/ disc) of endophytic fungi of *M. indica*. Diameter of inhibition zone (mm ± SD) of pathogenic bacteria. *A Enterococcus faecalis* (IMS/GN7), *B Escherichia coli* (ATCC 25922), *C Salmonella typhi* (MTCC 3216), *D Pseudomonas aeruginosa* (ATCC278553), *E Shigella boydii* (IMS/GN2), *F Salmonella enteritidis* (IMS/GN3), *G Morganella morganii* (IMS/GN6), *H Staphylococcus aureus* (ATCC 25923), *I Aeromonas hydrophila* (IMS/GN11) and *J Proteus mirabilis* (IMS/GN13)

Endophytic fungus	Isolate	A	B	C	D	E	F	G	H	I	J
Alternaria alternata	MIL3/1	0.00±0.00	7.00+0.00	9.67±1.00	NE[c]	0.00±0.00	0.00±0.00	0.00±0.00	6.33±0.58	5.75±0.58	0.00±0.00
Alternaria sp.	MIL7/2	0.00±0.00	0.00±0.00	9.33±1.00	NE	7.67±0.58	0.00±0.00	0.00±0.00	10.67±0.58	5.5±0.58	0.00±0.00
Aspergillus niger	MIB11/3	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00	0.00±0.00	0.00±0.00	0.00+0.00	0.00±0.00	0.00±0.00
A. fumigatus	MIB15/4	0.00±0.00	NE	11.67±1.00	NE	12.33±0.58	0.00±0.00	0.00±0.00	0.00+0.00	NE	0.00±0.00
Aspergillus sp.	MIL4/5	8.33+1.00	0.00±0.00	12±1.00	15.5±0.58	10.33±0.58	0.00±0.00	6.33±1.00	19.00±1.00	13±1.15	0.00±0.00
Aspergillus lentulus	MIB03/6	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00
Aspergillus tubingensis	MIB06/7	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00	NE	0.00±0.00	0.00±0.00	NE	0.00±0.00
Aschersonia sp.	MIL13/8	21.67±1.00	8.33±0.58	17.67±0.58	12.00±0.58	23.33±0.58	0.00±0.00	16.67±1.00	27.33±1.52	13.33±1.00	14.0±0.58
Botryosphaeria rhodina	MIB01/9	0.00±0.00	0.00±0.00	6.67±1.00	NE	25.33±1.53	13.33±1.00	7.00±1.00	NE	8.25±1.00	0.00±0.00
Botryodiplodia sp.	MIS05/10	6.67+1.00	0.00±0.00	NE	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	7.33±1.15	0.00±0.00	0.00±0.00
Cladosporium cladosporioides	MIS10/11	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00
Colletotricum gloeosporioides	MIS15/12	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Corynespora sp.	MIL20/13	11.67+1.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	10.33±0.58	NE	0.00±0.00
Emeriecella sp.	MIB02/14	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00
Fusarium sp.	MIB05/15	0.00±0.00	0.00±0.00	14±1.00	0.00±0.00	13.67±0.58	11.33±1.00	11.67±0.58	0.00±0.00	0.00±0.00	10.25±1.52
Fusarium sp.	MIS20/16	0.00±0.00	0.00±0.00	NE	NE	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00
Gliocladium sp.	MIL25/17	NE	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	7.00±1.00	NE	NE	0.00+0.00	0.00±0.00
Macrophoma sp.	MIL30/18	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	7.33±0.58	0.00±0.00	NE	7.00±1.00	NE	0.00±0.00
Penicillium sp.	MIB20/19	0.00±0.00	0.00±0.00	NE	NE	0.00±0.00	NE	0.00±0.00	0.00±0.00	NE	0.00±0.00
Pestalotia sp.	MIB25/20	0.00±0.00	11.33+0.58	0.00±0.00	0.00±0.00	0.00±0.00	7.67±1.00	0.00±0.00	0.00±0.00	0.00+0.00	0.00±0.00
Phomopsis sp.	MIS03/21	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Pleospora sp.	MIB04/22	0.00±0.00	9.00+1.00	0.00±0.00	0.00±0.00	0.00±0.00	10.33±1.00	13.67±1.00	17.33±0.58	NE	7.25±0.58
Pleospora sp.	MIB08/23	0.00±0.00	0.00±0.00	10±1.00	NE	0.00±0.00	0.00±0.00	NE	NE	NE	0.00±0.00
Xylaria sp.	MIB07/24	0.00±0.00	0.00±0.00	0.00±0.00	NE	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Methanol[a]	Control -ve	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Ciprafloxacin[b]	Control+ve	24.00±0.00	32.00±0.00	21.00±0.00	34.00±0.00	39.00±0.00	40.00±0.00	30.00±0.00	33.00±0.00	25.00±0.00	23.00±0.00

[a] Negative control

[b] Positive control and NE- Not evaluated

[c] Not evaluated

T-RFLP (Nikolcheva and Barlocher 2005), pyrosequencing and DGGE fingerprinting (Ovaskainen et al. 2010) have been used to clarify the taxonomic position of fungi. In the present study, 12 morphologically unidentified endophytic fungi were identified by ITS rDNA sequencing, and similar sequences were retrieved from the GenBank database for phylogenetic study (Table 1). The rDNA technique is now well known and has been employed successfully in the phylogenetic analysis and identification of morphologically unidentifiable endophytic fungi (Guo et al. 2001; Wang et al. 2005). Direct isolation of genomic DNA and identification of fungi from natural habitats has been reviewed by Liew et al (1998). This direct approach has also been employed for the analysis of endophytic fungal diversity (Tao et al. 2008); however, molecular identification strategies also fail in the absence of a high-quality reference database with broad coverage (Kharwar et al. 2009; Ovaskainen et al. 2010). The phylogenetic analysis based on molecular identification performed here revealed an interesting fact: only 3 out of 12 taxa identified shared more than 98 % similarity with known taxa in GenBank, while the remaining 75 % of species show lower proximity, which may indicate that studies such as this have an an important role to play in closing the gap between the undiscovered and discovered at the fungal diversity front.

The large sample size employed in this study allows us to draw significant conclusions [1,897 isolates from 2,700 tissue segments as compared to only 279 isolates from 2,400 segments in *Terminalia arjuna* and 79 isolates from 150 segments in *Aegle marmelos* and 407 isolates from *Nyctanthes arbor-tristis* in previous reports (Tejesvi et al. 2005; Gond et al. 2007, 2012)]. The dominance of *Phomopsis* sp. 1 in the present study is in accordance with the results of the Indian neem tree *Azadirachta indica* (Verma et al. 2007), while the dominance of *Fusarium* sp. and *Pestalotiopsis* sp. were similar to that of *Aegle marmelos* and *Terminalia arjuna*, respectively (Tejesvi et al. 2005; Gond et al. 2007). *Colletotrichum gloeosporioides* as the second dominant species in the present study is in accord with a study by Huang et al. (2008) conducted in 29 medicinal plants species. Finding the highest colonisation frequency in stem may be because of the spore abundance of a few dominant endophytic fungi in stem tissue. The ubiquitous species of fungal endophyte like *Aspergillus*, *Colletotrichum*, *Fusarium*, *Phomopsis*, *Penicillium* and *Trichoderma* recovered from *M. indica* (Table 2) have also been reported from leaves of *Menilkara bidentata*, which belongs to same family as *M. Indica* (Lodge et al. 1996). Recovery of genera like *Alternaria*, *Aspergillus*, *Chaetomium*, *Pestalotia* and *Cladosporium* in this study is in accordance with the well studied Indian plants *Aegle marmelos*, *Azadirachta indica*, *Terminalia arjuna*, *Eucalyptus*, *Adhatoda*, *Clerodendron*, *Bahunia* and *Adenocalymma alliaceum* (Tejesvi et al. 2005; Raviraja 2005; Verma et al. 2007; Gond et al. 2007; Kharwar et al. 2010, 2011b). Some species, like *Aspergillus tubingensis*, *A. lentulus*, *Preussia* sp. and *Fusicoccum* sp., were not reported as endophytes from the Indian subcontinent in previous studies, and the finding of these four rare/or accidental species in this experiment prove the authenticity and adequacy of the sampling method (Verma et al. 2007).

The overall maximum colonisation of endophytic fungi in the stem is supported by an earlier study (Verma et al. 2007). Colonisation in all tissues at Chandauli Loc 2 (Agricultural area) was lower than at BHU (Loc 1) and Hathinala (Loc 3). One possible reason may be the application of pesticides and horizontal sharing of endophytes by other plants in the agriculture fields of the Chandauli site; however, it seems that ecological conditions such as lower annual rainfall and comparatively low annual temperature may have strongly affected colonisation of host tissues by endophytes (Table 7).

The maximum number of coelomycete isolates recovered was significantly higher than that of hyphomycetes. This finding was really surprising and completely different from previous findings where hyphomycetes were reported as most prevalent endophytes, although the minimum isolation of ascomycetes was in accordance with earlier results (Tejesvi et al. 2005; Gond et al. 2007, 2012). The high occurrence (%CF) of coelomycetes may be due to the abundance of a few taxa like *Phomopsis* sp., *Colletotrichum* sp., *Macrophoma* sp. and *Pestalotia* sp. Tejesvi et al. (2005) also reported maximum isolation of *Pestlotiopsis* (coelomycetes) from *Terminalia arjuna*. The maximum recovery of coelomycetes from stems compared to other tissues at all the locations sampled could be because the specific phytochemistry of stems/twigs supports colonisation (Kharwar et al. 2008).

Table 7 Characteristics of Loc 1, Loc 2 and Loc 3

Site	Coordinates	Average temperature range	Average annual rainfall	Soil type
BHU (Loc1)	25°15′57″N 82°59′31″E	5–15 °C (in winter) 32–46 °C (in summer)	1,025 mm	Clay loamy soil
Chandauli (Loc2)	25°24′35″N 83°15′19″E	9–28 ° C (in winter) 22–40 °C (in summer)	869.5 mm	Sandy- clay loamy
Hathinala (Loc3)	24°18′5″N 83°5′58″E	2–15 °C (in winter) 32–42 °C (in summer)	1,075 mm	Sandy-Red soil

Overall diversity index (Shannon and Simpson) values are comparable to those found in previous studies in *Taxus globosa* (Orduña et al. 2011) and in *Azadirachta indica* (Verma et al. 2007), whereas the values found here are significantly higher than those in reports on the Indian medicinal plants *Clerodendron serratum*, *Callicarpa tomentosa*, *Adhatoda zeylanica* (Raviraja 2005) and *Terminalia arjuna* (Tejesvi et al. 2005).

The diversity of endophytes was highest in bark tissue, followed by leaf and stem twig. These results are in contrast to those found in *Tripterigium wilfordii* (Kumar and Hyde 2004) and *Nyctanthes arbor-tristis* (Gond et al. 2012), in which the highest Shannon index was reported in stem followed by leaf. However, in BHU, maximum diversity in bark tissue was ascribed to *Azadirachta indica* (Verma et al. 2007), which supports the present findings. The high diversity in bark samples can be explained by the longer exposure to fungi, moisture and air of bark compared to other tissues. Although stem tissue had maximum colonisation and isolation, it showed less diversity due to the dominance and uneven distribution of only a few taxa compared to other tissues. Of the three locations, Loc 1 showed maximum diversity, followed by Loc 2 and Loc 3. Verma et al. (2007) also found maximum diversity in *Azadirachta indica* at the BHU location. In *Eucalyptus citriodora*, an approximately equal Shannon index was observed from the Varanasi region (Kharwar et al. 2010).

The tissue specificity of endophytic fungi has been well documented in earlier studies. Specific assemblages of fungal endophytes in different tissues have been observed in *Acer macrophyllum* (Sieber and Dorworth 1994) and *Quercus pitracea* (Halmschlager et al. 1993). Our PCA analysis showed that 60 % of taxa were tissue specific—a significantly higher percentage than in *Taxus globosa* (Orduña et al. 2011) in which only 33.33 % of taxa showed tissue specificity. The occurrence of two *Pleospora* species only from the bark tissue is supported by the study of Orduña et al. (2011) in *Taxus globosa*. Endophyte tissue specificity has also been reported by many previous workers, which may be due to the varying ability to utilise resources from different host tissues (Rodrigues 1994; Photita et al. 2001; Cannon and Simmons 2002; Huang et al. 2008).

In antibacterial assays, 58.33 % of the isolates were found to be active (Table 6); in previous studies, only 8.3 % of isolates of *Dracaena cambodiana* and *Aquilaria sinensis* showed antimicrobial activity (Gong and Guo 2009), whereas 27.6 % isolates of *Camptotheca acuminata* displayed antimicrobial activity against some pathogens (Lin et al. 2007). However, contrary to this, some earlier findings reported that 75 % of the endophytic fungi from *N. arbor-tristis* and *Adenocalymma alliaecium* tested exhibited antibacterial activity (Gond et al. 2012; Kharwar et al. 2011b). It is interesting that five endophytic fungi, *Aschersonia* sp., *Aspergillus* sp., *Botryosphaeria rhodina*, *Pleospora* sp. and *Fusarium* sp. (MIB05), inhibited the growth of five or more than five out of ten tested human pathogens (50 %), revealing a broad spectrum of antibacterial activity (Table 6). Shu et al. (2004) reported cerebroside compounds with antibacterial and xanthine oxidase inhibitory activities from *Fusarium* sp. IFB-121 isolated from *Quercus variabilis*. Two important human pathogens, i.e. *Salmonella typhi* (the causative agent of typhoid fever) and *Staphylococcus aureus* (causing septicaemia) are very sensitive to many endophytic fungi, thus provide further impetus to further isolate the pure bioactive compounds. The zone of inhibition showed by *Aschersonia* sp. and *Aspergillus* sp. against *Staphylococcus aureus* is comparable to the strong antibiotic ciprofloxacin that was used as a positive control (Table 6). Several potent antimicrobial and cytotoxic compounds have been identified and characterized from endophytic *Chaetomium* spp., *Aspergillus fumigatus*, *A. niger*, *Alternaria* spp., *Botryodiplodia* spp., *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp., *Pestalotia* spp. and *Phomopsis* spp. isolated from different hosts (Kharwar et al. 2011a; Zhao et al. 2010; Kaul et al. 2013) and recovery of these endophytes also from this host opens up the exciting possibility of the further characterisation of their bioactive principles. Recently, two new antibacterial chromones, phomochromones A and B, and one new natural cyclopentenone derivative, phomotenone, have been reported from the endophytic fungus *Phomopsis* sp. isolated from host *Cistus monspeliensis* (Ahmed et al. 2011).

An endophytic *Alternaria* sp. isolated from the phloem of *Catharanthus roseus* has the ability to produce vinblastine and vincristine—both known anticancer compounds (Guo et al. 1998; Zhang et al. 2000). These results indicate that some endophytic fungi could be likely resources from which to produce different cytotoxic compounds. An antibacterial napthaquinone like javanicin was reported from an endophyte *Chloridium* sp. isolated from neem (Kharwar et al. 2009). Another interesting aspect of fungal endophytes is their production of antimicrobial volatile organic compounds (VOCs), as reported from the mitosporic xylariales fungi *Muscodor albus* and *M. vitigenus* isolated from *Cinamomum zeylanicum* (Strobel et al. 2001). Some antioxidant compounds, such as pestacin and isopestacin, are also reported from endophytic fungi.

Recently, some interesting works have been published aimed at enhancing the production of known and cryptic bioactive compounds through epigenetic modulation. Such studies may find a way in future to reduce the problem of reduced yield of fungal endophytes in successive generations (Sun et al. 2012; Hassan et al. 2012). The huge diversity of endophytic fungi (40 taxa) found in the present study, with significant antibacterial activity in the crude extracts of many of them, have provided us with a potential fungal endophyte pool from which to isolate pure and novel bioactive compounds—work that is already in progress in our laboratory (MMTL, BHU, Varanasi).

Acknowledgments The authors are thankful to the following: The Head and Coordinator, CAS in Botany, BHU, Varanasi, for facilities; Prof. Gopal Nath, Institute of Medical Sciences, BHU, Varanasi for antibacterial facility; the CSIR and UGC, New Delhi, for financial support as JRF and SRF; the DST, New Delhi, for financial support to RNK as project (File No. SR/SO/PS-78/2009, dt. 10-5-2010).

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