

# Diversity and bioactive potential of endophytic fungi from *Nothapodytes foetida*, *Hypericum mysorense* and *Hypericum japonicum* collected from Western Ghats of India

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**Abstract** Endophytic fungi are major contributors to fungal diversity and an important component of plant microbiota. Plants growing in biodiversity hotspots and having ethnobotanical utility are often explored for the presence of endophytic fungi with bioactive potential. Western Ghat mountains of India are one of the ten famous biodiversity hotspots in the world. Hence, in the present study, we investigated the diversity of endophytic fungi associated with tissues of *Nothapodytes foetida* (a tree), *Hypericum mysorense* (a shrub) and *Hypericum japonicum* (a herb) collected from forests of Western Ghats with emphasis on diversity of endophytic fungi harbored in different tissues of three plants and their antimicrobial and free radical scavenging activities. A total of 298 isolates belonging to 31 genera were isolated along with dark septate and sterile fungi. All isolates belonged to Dothidiomycetes, Eurotiomycetes and Sordariomycetes of the phylum Ascomycota. Most frequent colonizers were *Penicillium*, *Aspergillus*, *Fusarium*, *Gliocladium*, *Cladosporium*, *Trichoderma*, *Colletotrichum*, and *Pestalotiopsis*. Endophytes showed neither any host preference nor any dominance of a single species. Ethyl acetate extracts of 39 endophytic fungi exhibited antimicrobial activity against one or more pathogens, of which the activity of *Bionectria ochroleuca* NOTL33, *Chaetomium globosum* HYML55, *Alternaria brassicae* HYMS01, *Aspergillus* sp. HYML56 was prominent against most of the pathogens tested. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radicals

were effectively quenched by the ethyl acetate extracts of 28 isolates and 34 isolates, respectively. This study is the first of its kind in *H. mysorense* and *H. japonicum*, and not only describes the endophytic diversity of these plants but also emphasizes the bioactive potential of endophytic isolates for future use in agricultural, pharmaceutical and industrial applications.

**Keywords** Endophytic fungi · Western Ghats · ITS rDNA · DPPH · Antimicrobial · Diversity index · Antioxidant

## Introduction

Plant endophytes are a hyper-diverse community that make up a major component of plant microbiota. About 270,000 plant species on this planet are estimated to harbor about 13.6 million unique endophytic fungal species in addition to approximately 100,000 species of fungi already known to date (Kumar and Hyde 2004). Taking a broader perspective, endophytes are defined as microorganisms that spend all or part of their life cycle colonizing the healthy tissues of their host plants, either inter- or intra-cellularly, typically causing no apparent symptoms of disease (Zhao et al. 2010). An endophyte may be mutualistic, a latent pathogen or even a parasite inside the host. The ecological implications and role of endophytes in these mutualistic interactions have been studied in the case of the clavicipitaceous endophytes that inhabit grasses. The involved physiological interactions confer biotic stress tolerance on their hosts and help the host to adapt to different environmental conditions. Plant nutrition, plant survival and distribution are also influenced by endophytic communities (Qadri et al. 2014). However, such details are not available for non-clavicipitaceous fungi, which encompass virtually all the hyper-diverse endophytic population and are

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isolated ubiquitously from the tissues of higher plants (Rodríguez et al. 2009). Most studies on class 3 non-clavicipitaceous endophytic fungi have focused on exploring the bioactive metabolites that can be isolated from them (Rodríguez et al. 2009). The hypothetical distinction of endophytic biotopes and the probable genetic and metabolic transactions between the endophyte and host are thought to explain the production of myriad metabolites with functional and structural diversity. Thus endophytes are hypothesized to offer mutualistic benefits to their hosts by the production of antioxidant and antimicrobial secondary metabolites.

Plants growing in biodiversity hotspots or with an ethnobotanical background have been targeted for the isolation of endophytic fungi, as they are thought likely to yield hitherto unknown strains with novel bioactive compounds. However, an understanding of the real diversity of endophytes would be a primary prerequisite before exploring endophytic fungi for commercially important metabolites (Strobel 2014). Studying the diversity of endophytes from different plants collected from the same site could also address any discrepancies regarding the putative nature of endophytes. The endophytic composition of a plant clearly depends on host identity as well as on the geographic location of the host. The composition of endophytes in a monospecific host is affected strongly by local climatic conditions. The species richness of endophytes inhabiting plants in tropical rain forests outnumbers that of temperate forests, although less host specificity is seen (Hoffman and Arnold 2008).

The tropical forests of the Western Ghats of India are considered one of the ten major biodiversity hotspots of the world. In the present study, we elucidated the diversity of endophytic fungi from three medicinal plants—*Nothapodytes foetida* (a tree), *Hypericum mysorense* (a shrub) and *Hypericum japonicum* (a herb)—collected from the forests of Western Ghats of Karnataka, India. *Nothapodytes foetida* (Wt.) Sleumer (*Mappia foetida* Miers), Icacinaceae, is a medium-sized tree that grows wild in the forests of Western Ghats. The tree is a major source of important anti-neoplastic alkaloids such as camptothecin, 9-methoxycamptothecin, and 9-methoxy-20-O-acetylcampthothecin (Srinivas and Das 2003) exhibiting antimicrobial, antiviral and anticancer properties (Fulzele and Satdive 2005). *H. mysorense*—a perennial shrub growing luxuriantly in the cool climate of Western Ghats—was reported to produce many xanthone derivatives having antiviral and antioxidant activities. This plant is known in folklore medicinal preparations as spasmolytic, hypotensive and antibacterial. A number of phenolics and flavonoids have been reported from this plant, including hypericin, 2,3-hydroxyxanthone, 1,7-dihydroxyxanthone, 1-hydroxy-7-methoxyxanthone, 6,7-dimethoxy-1-hydroxyxanthone and 2-hydroxy-3-methoxyxanthone, hyperenone-B, mysorenone-B and mysorenone-C. Solvent extracts of this plant exhibit effective anticancer activity, antimicrobial activity, antiviral

activity and antioxidant activity (Moorthi et al. 2010). *Hypericum japonicum* Thunb. (Family: Hypericaceae) is an annual herb, called “Tianjihuang” in China, that is used widely for the treatment of bacterial diseases, infectious hepatitis, acute and chronic hepatitis, gastrointestinal disorder, internal hemorrhage and tumor. Different classes of chemicals, such as flavonoids, phloroglucinol derivatives, lactones, xanthonoids, chromone glycosides and peptides, have been reported in *H. japonicum*. Some bioactive chemicals like salothranols, saropyrone, salothralens, sarolactones, taxifolin-7-O-rhamnoside, isoquercitrin, quercitrin, chromone glycosides, quercetin and kaempferol have been characterized in *H. japonicum*. This plant is also known for its antioxidant properties (Samaga and Rai 2013a; Zuo et al. 2012).

There are no reports available on endophytic fungal diversity associated with *H. japonicum* or *H. mysorense*. The endophytic fungal biota of *N. foetida* have also not been studied comprehensively. Hence, this study attempts to evaluate the endophytic fungi of these plants, which may provide insights into their diversity, distribution and host affinity. An exploration of the bioactive potential of these endophytes may add to the applied and ecological perspectives of the endophytic community.

## Materials and methods

### Sampling

Small asymptomatic twigs of *Nothapodytes foetida* were collected from a full-grown tree near Alur, Hassan district, Karnataka, India (approximately 12° 94 N, 75° 89 E). *Hypericum mysorense* plants were collected from Talacauvery, Madikeri district, Karnataka, India (~12° 38 N, 75° 51 E). *Hypericum japonicum* plants were collected from Kigga, near Sringeri, Karnataka, India (~ 13° 41 N, 75° 19 E) and Bababudan Giri (~ 13° 52 N, 75° 74 E). All these sampling sites fall under the Western Ghat belt with similar climatic conditions. Sampling was carried out during July–October 2009. Samples were transported to the laboratory in sterile polypropylene plastic bags and processed within 24 h of collection.

### Isolation of endophytic fungi

Endophytic fungi were isolated from fresh disease-symptom-free samples following the protocol established previously by Samaga et al. (2014a). The samples were washed in water and surface sterilized by the following immersion sequence: 0.1 % mercuric chloride solution for 1 min, sterile water wash, 90 % ethanol for 2 min and sterile water wash. The leaf pieces (5 × 5 mm) and young stem pieces (5 mm) were placed on sterile water agar (pH 4.8, 1.5 % agar) and incubated at ambient

temperature ( $28 \pm 3$  °C) in the dark until hyphae emerged from the cut ends. These hyphae were subcultured on sterile potato dextrose agar (PDA, HiMedia, Mumbai, India). The isolates were pure cultured, sorted into morpho-species based on culture morphology, and pure cultures were maintained on PDA slants at 4 °C for further studies.

### Identification of isolates

Endophytic fungal isolates were identified up to genus level based on morphological features such as colony morphology, pigmentation, growth pattern, spore structures and other hyphal characteristics with the help of standard mycological manuals (Ellis 1971, 1976; Gilman 1971). Some abundant fungi common in all three medicinal plants were identified by sequencing of the internal transcribed spacer region (ITS1, 5.8S, ITS2) of ribosomal DNA.

### PCR amplification and sequencing of the ITS/5.8S genes

The isolates were grown for 6 days in potato dextrose broth (HiMedia) with added chloramphenicol (10 µg/mL). After the incubation, the fungal mat was separated by filtration and washed with sterile water to remove the residual antibiotic and exogenous metabolites. The fungal mats were then lyophilized and stored at  $-20$  °C. Lyophilized fungal mats were ground to a fine powder with liquid nitrogen using mortars and pestles. Genomic DNA was isolated from the ground mycelial mat with a Plant Genomic DNA Miniprep kit (HiMedia) following the instructions in the kit. The extracted DNA was stored at 4 °C until used.

Nuclear ribosomal ITS regions, which are considered a universal barcode marker for fungi, were amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (Amnion Biosciences, Bangalore, India) (White et al. 1990). The reaction volume was 25 µL, comprising 12.5 µL 2X PCR master mix (Merck Genei, Bangalore), 1 µL each of forward and reverse primers (10 pmol/µL), template DNA (12–30 ng/µL) and 9.5 µL sterile water. The amplification conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and final extension at 72 °C for 8 min. The amplicons were tested in agarose gel (1.2 %) electrophoresis and visualized using ethidium bromide (Sigma, Deisenhofen, Germany) staining.

### Sequence analysis and identification of endophytic isolates

The amplicons were sequenced by Sanger's dideoxy nucleotide chain termination method using ITS1 primer for forward sequencing (Amnion Biosciences, Bangalore, India). Sequences were deposited with the NCBI-GenBank database

and accession numbers obtained. These sequences were used as the queries for similarity searches in the GenBank database using the mega-BLAST algorithm. The isolates were identified based on the best hit in the similarity search using BLAST. In case of multiple matching sequences, identity was confirmed by phylogenetic analysis. For this, the best hits for a query sequence were downloaded from the GenBank database, aligned using the ClustalW online tool utilizing default settings. The phylogenetic tree was generated using MEGA 6 (Tamura et al. 2011) by the maximum parsimony (MP) method. A bootstrap consensus tree was inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. The MP tree was obtained using the subtree-pruning-regrafting with search level 1, in which the initial trees were obtained with the random addition of sequences (ten replicates).

### Diversity assessment using statistical indices

The magnitude of endophytic assemblages in plant tissues was estimated on the basis of isolation frequency (IF) given by the following formula (Qadri et al. 2014):

$$IF = \left[ \frac{\text{Number of isolates obtained}}{\text{Total number of tissue segments placed}} \right] \times 100$$

Diversity of the endophytic fungal community was assessed using various statistical indices. The proportion of individual taxa in the total number of isolates was assessed on the basis of colonization frequency (CF) (Qadri et al. 2014), which was given by:

$$CF = \left[ \frac{\text{Number of isolates of one taxon}}{\text{total number of isolates}} \right] \times 100$$

The biodiversity of endophytic fungi was analyzed by Shannon-Weaver index (H) (Shannon and Weaver 1949) and Simpson index (1-D) (Simpson 1949). Dominance was calculated with Simpson's dominance (D) and Berger Parker dominance (d) (Caruso et al. 2008) measures. The formulae were as follows;

$$H = -\sum (Pi)(\ln Pi)$$

$$D = \sum (Pi)^2$$

Where, the ratio "Pi" is the frequency of colonization of the taxon in the sample.

$$d = N_{max}/N$$

Where,  $N_{max}$  is the number of individuals in the most abundant species, and N is the total number of individuals in the sample.

True diversity is given by the effective species numbers. Fischer's alpha ( $\alpha$ ), (Fisher et al. 1943), is one such parametric index. Similarly, the effective species number also can be deduced by the exponent of the Shannon entropy value (Jost 2006). The formulae are:

$$S = \alpha \times \ln\left(1 + \left(\frac{n}{\alpha}\right)\right)$$

Where 'S' is number of taxa, 'n' is number of individuals and ' $\alpha$ ' is Fischer's ' $\alpha$ '

$$\text{Effective species no.} = e^H$$

Where ' $H$ ' is the Shannon index.

Evenness index (J) was used for the determination of uniformity of the endophytic fungi (Zheng et al. 2013), which was given by:

$$J = \frac{H}{\ln(s)}$$

Where, 'S' is the total number of fungi isolated.

The Sorenson ( $C_s$ ) and Jaccard ( $C_j$ ) indices were employed to determine the similarity coefficient between the two locations of endophytic fungi (Zheng et al. 2013). The formulae are:

$$C_s = \frac{2j}{a+b} \quad C_j = \frac{j}{a+b-j}$$

Where, 'j' is the total number of species or genera common to both samples, 'a' and 'b' are the number of species or genera in plots 'A' and 'B', respectively.

The calculations were performed using Microsoft Excel spreadsheet functions and the paleontological statistics software package known as PAST 3 (Harper et al. 2001). The significance difference among the endophytic diversity of samples was calculated by pair-wise Hutchinson's diversity - t test using the PAST 3 tool.

### Fermentation and extraction of secondary metabolites

All endophytic isolates were screened initially for antimicrobial activity employing the agar plug method as described previously (Larsen and Knöchel 1997). Isolates showing promising antimicrobial activity in the preliminary screening were tested further in a secondary screening, during which metabolites were extracted from the culture medium and tested for activity (Samaga et al. 2014a).

### Evaluation of antimicrobial activity

Microbial strains used in the study were obtained from Institute of Microbial Technology (IMTECH), Chandigarh.

Test strains used were *Pseudomonas aeruginosa* (MTCC 7093), *Enterobacter aerogenes* (MTCC 111), *Shigella flexneri* (MTCC 1457), *Bacillus subtilis* (MTCC 121), *Salmonella enterica* ser. Typhi (MTCC 733), *Staphylococcus aureus* (MTCC 7443), *Streptococcus pyogenes* (MTCC 1925), *Candida albicans* (MTCC183), *Microsporium gypseum* (MTCC 2830), and *Microsporium canis* (MTCC 2831).

Antimicrobial assays were carried out using the disc diffusion method (Bauer et al. 1966). Ethyl acetate extracts were tested at 400  $\mu\text{g}/\text{disc}$  concentrations (loaded in 40  $\mu\text{L}$  aliquots). Discs loaded with solvent alone (40  $\mu\text{L}$ ) were used as a negative control. Chloramphenicol (HiMedia, 30  $\mu\text{g}/\text{disc}$ ) and Nystatin (HiMedia, 30  $\mu\text{g}/\text{disc}$ ) discs were used as positive controls for bacteria and fungi, respectively. The inhibition zones around the discs were measured using a zone scale (HiMedia). The experiment was carried out in triplicate.

### Evaluation of free radical scavenging activity

#### DPPH radical scavenging assay

Two-fold dilutions of extracts in the range from 5 to 0.039 mg/mL in methanol (100  $\mu\text{L}$ ) were mixed with 100  $\mu\text{L}$  DPPH solution (1,1-diphenyl-2-picrylhydrazyl, Sigma, 40  $\mu\text{mol}/\text{L}$ ). A control was maintained by adding 100  $\mu\text{L}$  DPPH to 100  $\mu\text{L}$  methanol. The plates were incubated for 30 min in the dark at 25 °C. The decrease in absorbance was measured at 517 nm after the incubation and the activity was calculated using the formula:

DPPH scavenging activity (%)

$$= \left[ \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \right] \times 100$$

The assay was carried out in triplicate. Antioxidant activity was expressed as milligram equivalents of ascorbic acid (Samaga and Rai 2013b).

#### ABTS radical scavenging assay

The free radical scavenging activity of the crude extracts was determined by using 2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) stable cationic free radicals (Samaga et al. 2013b). The ABTS free radical solution was prepared by reacting 3.75 mM ABTS diammonium salt with 1.225 mM potassium persulphate solution at 30 °C. A 200- $\mu\text{L}$  aliquot of the standardized ABTS solution (absorbance adjusted to  $0.6 \pm 0.05$  at 734 nm) was mixed with various concentrations of the extract in 10  $\mu\text{L}$  aliquots (5 to 0.039 mg/mL in two-fold dilutions in methanol) and the absorbance was

read at 734 nm every 5 min up to 60 min. The ABTS quenching activity was calculated using the formula:

ABTS scavenging activity (%)

$$= \left[ \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \right] \times 100$$

The assay was carried out in triplicate. The concentration required for a 50 % reduction in ABTS radical ( $IC_{50}$ ) was determined graphically. The antioxidant capacity was expressed as milligram equivalents of ascorbic acid (AA).

## Results

### Diversity and phylogenetics of endophytic fungi

The abundance of endophytic fungi associated with tissues from *N. foetida*, *H. mysorensis* and *H. japonicum* was quantified by measuring the IF. A total of 298 fungal strains was isolated from 378 tissue segments of the three medicinal plants. IFs of endophytic fungi from leaves and stem segments of *N. foetida* were 74 % and 76.67 %, respectively. *H. mysorensis* leaves were richer than the stems in terms of endophytic fungal infestations as reflected by their IF values of 98 % and 69.39 %, respectively. *H. japonicum* stems also showed a higher IF of 84 %.

Based on morphological features, the isolates were identified at least up to genus level and those fungal strains that could not be identified by morphological features were segregated into morphospecies. Endophytic strains failing to produce any spores in spite of efforts to make them sporulate were considered as sterile morphospecies. Sterile morphospecies with dark hyphae were noted separately as dark septate endophytes (DSE).

All identified isolates were Ascomycete members, represented by 31 genera, belonging to 16 families segregated under three major classes: Dothidiomycetes, Eurotiomycetes and Sordariomycetes (Table 1, Fig. 1). Major genera identified collectively from all the three plants were *Acremonium*, *Alternaria*, *Aspergillus*, *Bionectria*, *Bipolaris*, *Botryosphaeria*, *Cephalosporium*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Cylindrocarpon*, *Cylindrotrichum*, *Fusarium*, *Gibberella*, *Gliocladium*, *Lasiodiplodia*, *Monodictys*, *Myrothecium*, *Nigrospora*, *Penicillium*, *Pestalotiopsis*, *Peyronellaea*, *Phoma*, *Phomopsis*, *Stachylidium*, *Talaromyces*, *Trichoderma*, *Vermiculariopsisella*, *Verticillium* and *Xylaria*. Dark septate endophytes (DSE) and sterile morpho-species were collectively considered as uncategorized. The colonization frequencies of individual isolated taxa are tabulated in Table 1.

The number of taxa obtained from each of the tissues infers the species richness of the respective tissues. *N. foetida* leaves harbored 33 taxa, whereas the stems yielded only 11 taxa of

endophytic fungi. The leaves and stem segments of *H. mysorensis* yielded 30 and 39 taxa, respectively. *H. japonicum* stem segments harbored in total 33 fungal taxa. Species belonging to the genera *Colletotrichum*, *Fusarium* and *Gliocladium* (*Bionectria*) were commonly isolated from all tissues of *N. foetida*, *H. mysorensis* and *H. japonicum*. *Alternaria* sp., *Curvularia* sp., *Cylindrotrichum* sp., *Verticillium* sp., *Vermiculariopsisella* sp., and *Nigrospora* sp. were common to at least two of each of the medicinal plants. The genera *Aspergillus*, *Cladosporium*, *Myrothecium*, *Penicillium*, *Pestalotiopsis*, *Phoma* and *Phomopsis* were commonly isolated from all other tissues except the stems of *N. foetida*. Some taxa were found exclusively in particular tissues. Only the leaves of *N. foetida* harbored *Monodictys* sp. and *Xylaria* sp. Similarly, *Chaetomium globosum* and *Stachylidium* sp. were isolated only from leaves of *H. mysorensis*. *Peyronellaea* sp. was found only in the stems of *H. japonicum*; *Talaromyces* sp. was isolated only from *H. mysorensis* stem segments; *Acremonium* sp., *Cylindrocarpon* sp., and *Trichoderma* sp. were exclusive to *N. foetida* and *H. japonicum* tissues.

Thirty isolates were identified by ITS DNA sequence analysis. The ribosomal DNA sequences of these isolates (partial 18S-ITS1-5.8S-ITS2-28S partial) were submitted to the GenBank database of NCBI and accession numbers were obtained (Table 2). A total 30 fungal strains of 15 taxa chosen from the total endophytic fungi isolated from all three medicinal plants was identified. The phylogenetic relationships of these 30 representative genera of endophytes were deduced by constructing a maximum parsimony tree (supported by 1000 bootstrap replications) using these ITS sequences (Fig. 2). The isolates were clustered into six major clades, each of which was further divided into sub-clades except the clade of *Phomopsis* sp.

The diversity index values (Table 3) suggest that the tissues of *N. foetida*, *H. mysorensis* and *H. japonicum* were highly diverse but distributed evenly. A lowest value of the Shannon–Weiner index was recorded in *N. foetida* stems (2.172) and *H. mysorensis* stems recorded a highest value (5.08) among the tissues studied. Fischer's  $\alpha$  values also depict that *H. mysorensis* stems have highest effective species numbers and *N. foetida* stems have the lowest numbers of effective species. Evenness and equitability indices were highest in *N. foetida* leaves and lower in stems of *N. foetida* and *H. japonicum*.

The endophytic diversity between leaves and stems of *N. foetida* and *H. mysorensis* was quantified by Sorenson's index ( $C_s$ ) and Jaccard coefficients ( $C_j$ ). The diversity of endophytes among different plant tissues selected for the study was analyzed by Hutchinson's Diversity  $t$  test. The lower  $C_s$  and  $C_j$  values of 0.318 and 0.189, respectively, recorded in case of the endophytic diversity of leaves and stems of *N. foetida* implies a greater difference in the endophytic

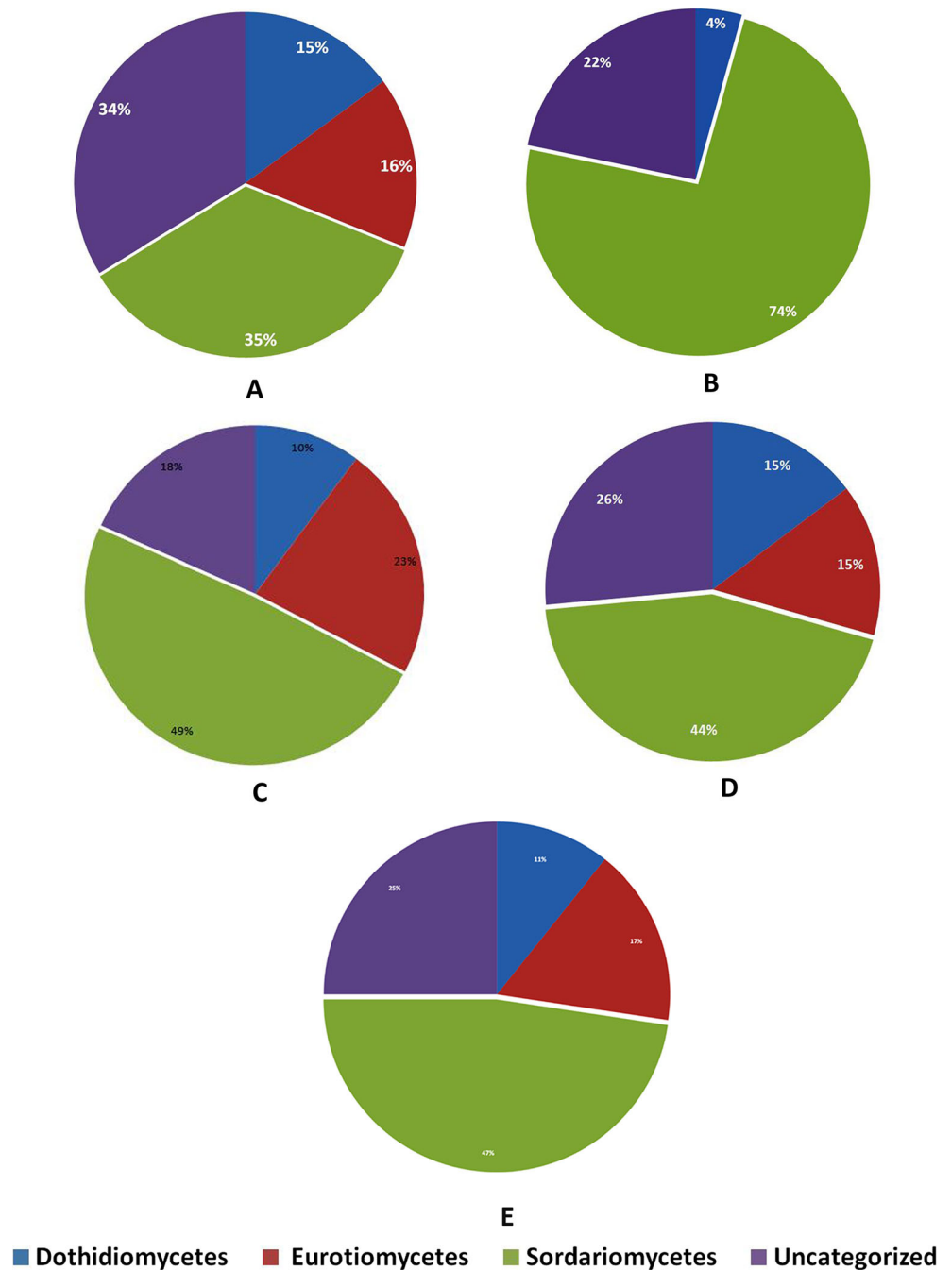
**Table 1** Endophytic taxa (colonization frequency) isolated from tissues of *Nothapodytes foetida*, *Hypericum mysorensense* and *Hypericum japonicum*. CF Colonization frequency, DSE Dark septate endophytes

Class	Family	Endophytes	<i>Nothapodytes foetida</i> CF(%)		<i>Hypericum mysorensense</i> CF(%)		<i>Hypericum japonicum</i> CF(%)		
			Leaves	Stem	Leaves	Stem	Leaves	Stem	
Dothidiomycetes	Davidiellaceae	<i>Cladosporium herbarum</i>	0	0	0	1.35	0	0	
		<i>Cladosporium oxysporum</i>	1.35	0	1.35	0	5.41	0	
		<i>Cladosporium sphaerospermum</i>	1.35	0	0	1.35	1.35	0	
	Botryosphaeriaceae	<i>Botryosphaeria</i> sp.	1.35	0	0	1.35	0	0	
		<i>Lasioidiplodia theobromae</i>	0	0	1.35	2.70	0	0	
	Incertae sedis	<i>Monodictys</i> sp.	2.70	0	0	0	0	0	
		<i>Phoma</i> sp.	2.70	0	2.70	2.70	0	0	
	Pleosporaceae	<i>Alternaria alternata</i>	1.35	0	0	0	0	0	
		<i>Alternaria brassicae</i>	0	0	0	1.35	2.70	0	
		<i>Helminthosporium</i> sp.	1.35	0	0	0	0	0	
		<i>Bipolaris hawaiiensis</i>	0	0	1.35	0	0	0	
		<i>Curvularia lunata</i>	2.70	0	0	0	0	0	
		<i>Curvularia clavata</i>	0	4.34	0	0	0	0	
	Eurotiomycetes	Trichocomaceae	<i>Curvularia catenulata</i>	0	0	0	1.35	0	0
			<i>Curvularia pallescens</i>	0	0	0	1.35	0	0
			<i>Penicillium funiculosum</i>	3	0	2.70	0	0	0
<i>Penicillium chrysogenum</i>			0	0	1.35	2.70	1.35	0	
<i>Penicillium notatum</i>			0	0	2.70	0	0	0	
<i>Penicillium</i> Morpho species 1			2.70	0	0	1.35	2.70	0	
<i>Penicillium</i> Morpho species 2			1.35	0	0	0	6.76	0	
<i>Aspergillus calidoustus</i>			0	0	0	1.35	0	0	
<i>Aspergillus ustus</i>			0	0	0	1.35	0	0	
<i>Aspergillus nidulans</i>			2.70	0	1.35	0	0	0	
<i>Aspergillus tamari</i>			2.70	0	0	0	0	0	
<i>Aspergillus versicolor</i>			0	0	0	1.35	0	0	
<i>Aspergillus</i> morpho species 1			2.70	0	1.35	1.35	2.70	0	
<i>Aspergillus</i> morpho species 2			0	0	1.35	1.35	2.70	0	
<i>Aspergillus</i> morpho species 3			0	0	1.35	0	1.35	0	
Sordariomycetes			Amphisphaeriaceae	<i>Nigrospora sphaerica</i>	0	0	2.70	1.35	1.35
	<i>Talaromyces pinophilus</i>	0		0	0	1.35	0	0	
	<i>Pestalotiopsis</i> sp.	4.05		0	1.35	6.76	1.35	0	
	<i>Pestalotiopsis mangiferae</i>	0		0	0	4.05	0	0	
	<i>Cylindrotrichum</i> sp.	1.35		0	0	1.35	2.70	0	

Table 1 (continued)

Class	Family	Endophytes	Nothapodytes foetida CF(%)		Hypericum mysorense CF(%)		Hypericum japonicum CF(%)	
			Leaves	Stem	Leaves	Stem	Leaves	Stem
Dipterocarpaceae	Dipterocarpaceae	<i>Chaetomium globosum</i>	0	0	1.35	0	0	0
		<i>Phomopsis</i> sp.	6.76	0	2.70	4.05	1.35	1.35
		<i>Colletotrichum gloeosporoides</i>	1.35	8.69	2.70	4.05	5.41	5.41
		<i>Vermiculariopsis</i> sp.	1.35	0	1.35	0	0	0
		<i>Acremonium</i> sp. 1	0	21.74	0	0	2.70	2.70
		<i>Cephalosporium</i> sp.	0	8.69	0	1.35	0	0
		<i>Trichoderma</i> sp.	0	21.74	0	0	14.86	14.86
		<i>Myrothecium</i> sp.	2.70	0	2.70	1.35	2.70	2.70
		<i>Verticillium</i> sp.	0	0	0	1.35	1.35	1.35
		<i>Fusarium</i> morpho species 1	2.70	4.34	2.70	1.35	1.35	1.35
		<i>Fusarium</i> morpho species 2	2.70	4.34	1.35	0	0	0
		<i>Fusarium verticilloides</i>	0	0	0	4.05	2.70	2.70
		<i>Fusarium chlamydosporum</i>	0	0	0	1.35	0	0
Nectriaceae	Nectriaceae	<i>Fusarium phyllophylum</i>	0	0	0	0	1.35	1.35
		<i>Fusarium sporotrichioides</i>	0	0	0	4.05	0	0
		<i>Cylindrocarpon</i> sp.	0	0	0	0	4.05	4.05
		<i>Gibberella moniliformis</i>	2.70	0	1.35	0	0	0
		<i>Gliocladium catenulatum</i>	2.70	0	4.05	1.35	1.35	1.35
		<i>Gliocladium roseum</i>	5.41	4.34	5.41	2.70	9.46	9.46
		<i>Gliocladium virens</i>	0	0	0	0	1.35	1.35
		<i>Gliocladium viride</i>	0	0	1.35	1.35	0	0
		<i>Cylindrocarpon</i> sp.	1.35	0	0	0	0	0
		<i>Stachyidium</i> sp.	0	0	2.70	0	0	0
		DSE	6.76	0	2.70	10.81 (3 morpho species)	6.76 (2 morpho species)	6.76 (2 morpho species)
		Uncategorized	27.03 (5 morpho species)	21.74 (3 morpho species)	9.46 (3 morpho species)	13.51 (4 morpho species)	21.62 (4 morpho species)	21.62 (4 morpho species)

**Fig. 1 a–e** Class-wise proportion (%) of endophytic isolates from leaves (a) and stems (b) of *Nothapodytes foetida*, leaves (c) and stems (d) of *Hypericum mysorens*, and stems of *Hypericum japonicum* (e)



assemblages between these tissues. The diversity of endophytes from tissues of *H. mysorens* recorded a higher  $C_s$  and  $C_j$  values of 0.667 and 0.523, respectively, suggesting more than 50 % similarity of the endophytic composition between leaves and stems of this plant. The endophytic composition of only *N. foetida* stems significantly differ from all other tissues ( $P < 0.05$ ). The diversity of endophytic composition of all other tissues did not differ significantly from each other as deduced by the Hutchinson's diversity  $t$  test.

#### Antimicrobial activity of endophytic fungal extracts

All 298 endophytic fungi isolated from tissues of *N. foetida*, *H. mysorens* and *H. japonicum* were subjected to a preliminary screening for antimicrobial activity by employing the agar plug diffusion method. Endophytic fungal strains that produced inhibitory zones of not less than 15 mm against at least one of the test bacteria was considered for further fermentation and secondary screening by the paper disc diffusion method. A total of 39 endophytic fungi was selected for



**Table 2** Accession numbers of ribosomal DNA sequences of endophytic isolates submitted to GenBank

Sample no.	Accession code	Organism name
1	JX857165.1	<i>Alternaria brassicae</i> HYMS01
2	JX857166.1	<i>Pestalotiopsis</i> sp. HYMS02
3	JX857167.1	<i>Lasiodiplodia theobromae</i> HYMS42
4	JX857168.1	<i>Pestalotiopsis mangiferae</i> HYMS64
5	JX500352.1	<i>Chaetomium globosum</i> HYML55
6	JX500353.1	<i>Cladosporium sphaerospermum</i> NOTL67
7	JX500354.1	<i>Aspergillus calidoustus</i> HYMS46
8	JQ794833.1	<i>Bionectria ochroleuca</i> NOTL33
9	JQ794834.1	<i>Aspergillus</i> sp. HYML56
10	KC342043.1	<i>Bionectria ochroleuca</i> NOTL18
11	KC342044.1	<i>Phomopsis</i> sp. NOTL16
12	KC342045.1	<i>Colletotrichum gloeosporioides</i> HYMS26
13	KC342046.1	<i>Talaromyces pinophilus</i> HYMS04
14	KC342047.1	<i>Talaromyces funiculosus</i> NOTB11
15	KC342048.1	<i>Gibberella moniliformis</i> NOTL81
16	KC342049.1	<i>Gibberella moniliformis</i> NOTL84
17	KC342050.1	<i>Gibberella moniliformis</i> HYML20
18	KF180161.1	<i>Colletotrichum gloeosporioides</i> HYJ10S
19	KF180162.1	<i>Colletotrichum gloeosporioides</i> HYJ25
20	KF180163.1	<i>Colletotrichum gloeosporioides</i> HYJ26
21	KF180164.1	<i>Colletotrichum gloeosporioides</i> HYML53
22	KF180165.1	<i>Bionectria ochroleuca</i> HYML35
23	KF180166.1	<i>Bionectria ochroleuca</i> HYJ30
24	KF736447.1	<i>Bionectria ochroleuca</i> HYML22
25	KF736448.1	<i>Bionectria ochroleuca</i> HYML24
26	KF736449.1	<i>Bionectria ochroleuca</i> HYJE29
27	KF736450.1	<i>Bionectria ochroleuca</i> HYJE31
28	KF736451.1	<i>Bionectria ochroleuca</i> NOTL57
29	KF736452.1	<i>Bionectria ochroleuca</i> HYJE74
30	KF736453.1	<i>Bionectria ochroleuca</i> NOTL86

secondary screening based on the above criteria. Total of 17 isolates from *N. foetida* (17.58 %), 14 isolates from *H. mysorensis* (11.96 %) and 8 isolates from *H. japonicum* (9.52 %) showed such detectable activity and were selected for further fermentation and extraction (Table 4). Ethyl acetate total extracts from the selected isolates were tested for antimicrobial activity by paper disc diffusion assay. Out of these 39 endophytic strains, 10 isolates were sterile mycelia, constituting 25.64 % of the total bioactive strains. Apart from these, another 29 bioactive strains belonged to 15 different genera, viz. *Acremonium*, *Alternaria*, *Aspergillus*, *Bionectria*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Cylindrocarpon*, *Fusarium*, *Lasiodiplodia*, *Penicillium*, *Pestalotiopsis*, *Phomopsis*, and *Trichoderma*.

*Salmonella enterica* ser. Typhi was inhibited by most of the isolates and *Pseudomonas aeruginosa* remained most

resistance towards the action of the endophytic fungal extracts. Only a few endophytic strains, such as *Chaetomium globosum*, *Bionectria ochroleuca*, *Colletotrichum gloeosporioides*, *Alternaria* spp., *Pestalotiopsis* spp., *Penicillium* sp. and *Fusarium verticilloides* had shown anti-candidal and antidermatophytic activity, of which *B. ochroleuca* NOTL33, *Chaetomium globosum* HYML55 and *Pestalotiopsis* spp. (HYMS - 02, 64, 65, 66, 93) exhibited prominent anticandidal and antidermatophytic activity.

### Free radical scavenging activity

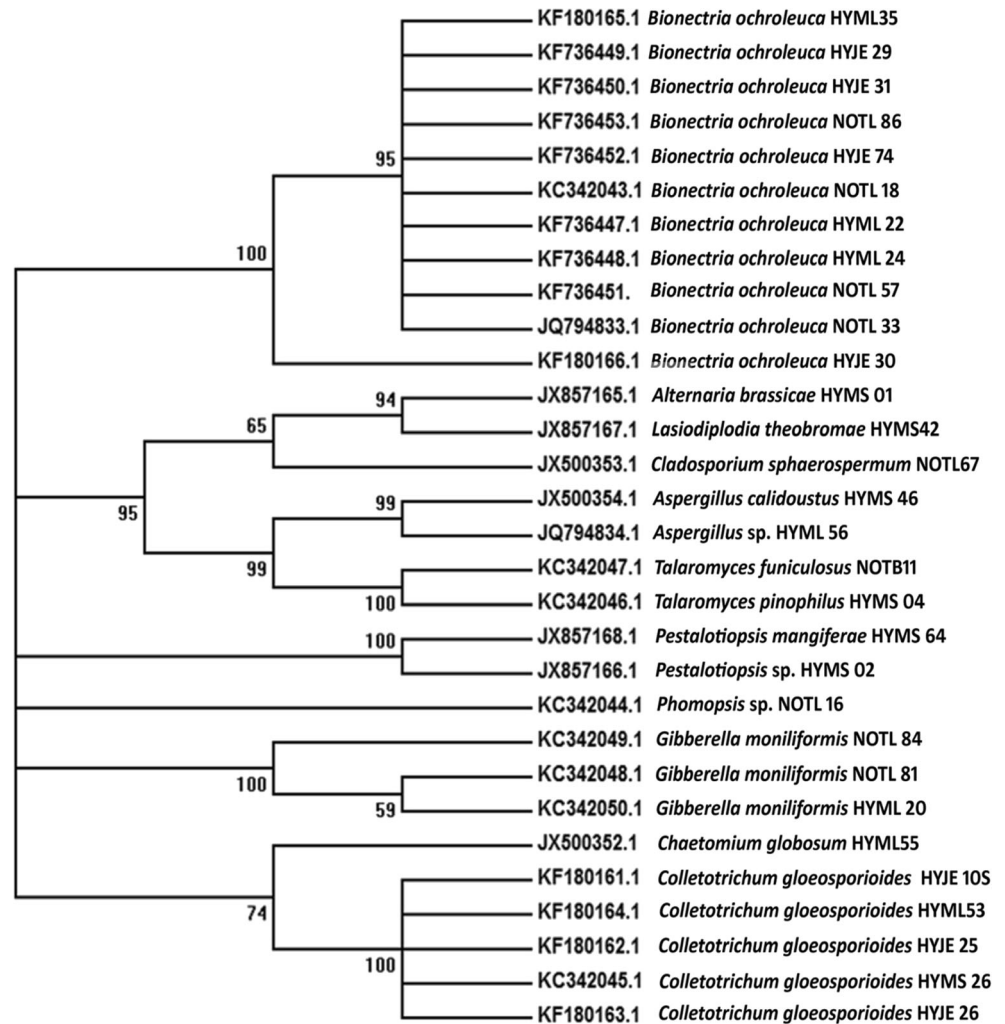
#### DPPH radical scavenging activity

Endophytic fungal isolates failing to quench 50 % of DPPH radicals during the preliminary screening at a concentration of 5 mg/mL were not considered for further study. Ethyl acetate extracts of nine isolates of *N. foetida*, 11 isolates of *H. mysorensis* and eight strains of *H. japonicum* exhibited effective quenching of DPPH free radicals. A dose-dependent radical quenching was observed (Fig. 3). Of the total number of tested isolates, 55 % exhibited more than 95 % quenching of DPPH radicals and 40 % recorded more than 75 % scavenging activity. The concentration required for 50 % quenching of free radicals represents the significance of the activity. The activity of strains *Talaromyces pinophilus* HYMS04, *Penicillium* sp. HYMS16, sterile mycelia NOTB11, *Acremonium* sp. NOTB29 and *Gibberella moniliformis* NOTL84 were highly significant ( $P \leq 0.001$ ) as depicted by their lower  $IC_{50}$  values (data not shown).

#### ABTS radical scavenging activity

Endophytic fungal extracts with less than 50 % ABTS radical scavenging at the concentration of 1 mg/mL were discarded during the preliminary screening and not considered for the further study. A total of 12 isolates each from *N. foetida* and *H. mysorensis* and ten isolates from *H. japonicum* had exhibited effective ABTS scavenging. ABTS radical scavenging activity was dose dependent (Fig. 4). A total of 66.67 % of the tested isolates recorded more than 95 % scavenging of ABTS radicals and 29.6 % showed more than 75 % activity. The strains *Alternaria brassicae* HYMS01, *Talaromyces pinophilus* HYMS04, *Lasiodiplodia theobromae* HYMS42, *Curvularia pallescens* HYMS94, sterile morphospecies (NOTB11 and NOTB14), *Acremonium* sp. NOTB29, and *Cladosporium sphaerospermum* NOTL67 were exhibited maximum ABTS quenching.

**Fig. 2** Phylogenetic tree of the selected endophytic taxa isolated from *N. foetida*, *H. mysorensis* and *H. japonicum*, where evolutionary history was inferred using the maximum parsimony (MP) method tested with 1000 bootstrap replications. All positions with less than 5 % site coverage were eliminated. The analysis involved 30 nucleotide sequences and 362 positions in the final dataset. Evolutionary analysis was conducted using MEGA 6



## Discussion

Mutualistic endophytes influence their hosts in various ways. Apart from imparting resistance against pests, insects,

pathogens and animals, they also modulate host metabolism. The biosynthetic capacity of endophytes is also influenced by their hosts which, in turn, results in the production of novel metabolites. Thus, endophytes play a major ecological role.

**Table 3** Assessment of diversity of endophytic biota using statistical indices

Statistics	<i>N. foetida</i>		<i>H. mysorensis</i>		<i>H. japonicum</i>
	Leaves	Stem	Leaves	Stem	Stem
Simpson's dominance index (D)	0.04164	0.1380	0.04040	0.03830	0.05205
Berger parker index (d)	0.09459	0.2174	0.08163	0.07353	0.13410
Simpson's diversity index (1-D)	0.95840	0.8620	0.95960	0.96140	0.94790
Shannon Index (H)	3.32000	2.1720	3.30500	5.08200	3.21500
Equitability	0.95800	0.9058	0.97160	0.96140	0.92760
Evenness (J)	0.86460	0.7977	0.90790	0.86820	0.77820
Fischer alpha	21.4200	8.2710	32.8800	38.0500	19.3000
Effective species no. ( $e^H$ )	27.6600	15.059	27.2400	33.8500	25.8200
Sorenson's index	0.318182		0.666667		NA <sup>a</sup>
Jaccard's coefficient	0.189189		0.5227		NA <sup>a</sup>

<sup>a</sup> Not applicable

**Table 4** Screening of endophytes from *N. foetida* stems for antimicrobial activity. NOTL *N. foetida* leaves, NOTB *N. foetida* stems, HYML *H. mysorensis* leaves, HYMS *H. mysorensis* stems, HYJE *H. japonicum* stems

Fungal endophyte	Pathogens used in the antimicrobial screening												
	<i>Escherichia coli</i>	<i>Enterobacter aerogenes</i>	<i>Bacillus subtilis</i>	<i>Klebsiella pneumoniae</i>	<i>Shigella flexneri</i>	<i>Salmonella enterica</i> ser. Typhi	<i>Staphylococcus aureus</i>	MRSA <sup>b</sup>	<i>Pseudomonas aeruginosa</i>	<i>Candida albicans</i>	<i>Microsporium gypsum</i>	<i>Microsporium canis</i>	
Sterile morphospecies NOTB 11	+	+	++	-	++	+	+	-	-	-	-	-	
Sterile morphospecies NOTB14	+	-	-	-	-	+	+	+	-	-	-	-	
<i>Acremonium</i> sp. NOTB29	-	-	-	-	-	+	++	-	+	+	+	+	
Sterile morphospecies NOTB14	+	+	++	++	++	++	++	-	-	-	-	-	
Sterile morphospecies NOTL11	+	+	++	-	++	+	-	-	++	++	++	++	
Sterile morphospecies NOTL12	+	+	-	+	-	-	-	+	-	-	-	-	
<i>Alternaria alternata</i> NOTL26	-	+	-	-	-	-	++	-	++	++	+	+	
<i>Bionectria ochroleuca</i> NOTL 33	+++	+++	+++	+++	+++	+++	++	-	+++	+++	+++	+++	
<i>Phomopsis</i> sp. NOTL37	+++	+++	-	-	-	++	+++	-	+	+	+	+	
<i>Phomopsis</i> sp. NOTL42	-	+	-	-	-	-	+	+	+	+	+	+	
<i>Cladosporium oxysporum</i> NOTL43	+	+	-	-	-	-	+	-	-	-	-	-	
<i>Colletotrichum gloeosporoides</i> NOTL46	-	-	-	-	-	-	-	++	-	-	-	-	
<i>Cylindrocarpum</i> sp. NOTL72	+	-	+	-	++	+	ND	++	-	-	-	-	
<i>Penicillium funiculosum</i> NOTL74	-	-	+	-	+	-	+	-	-	-	-	-	
Sterile morphospecies NOTL79	++	++	-	++	++	++	++	++	-	-	-	-	
<i>Aspergillus</i> sp. NOTL91	+++	++	++	+++	+++	+++	-	+++	-	-	-	-	
Sterile morphospecies NOTL92	-	-	-	-	-	++	+	+	+	+	+	+	
<i>Alternaria brassicae</i> HYMS 01	+++	++	++	+++	+++	+++	-	-	++	++	++	++	
<i>Pestalotiopsis</i> sp. HYMS02	+++	+	++	++	++	++	+	-	++	+	+	+	
<i>Colletotrichum gloeosporoides</i> HYMS11	+	+	-	-	-	-	-	+	+	-	-	-	
-	-	-	+	+	+	+	+	-	+	+	+	+	

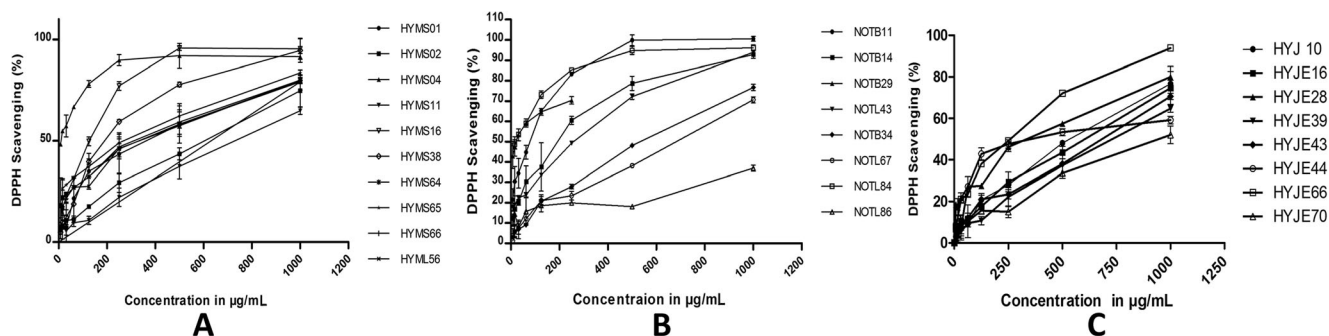
Table 4 (continued)

Fungal endophyte	Pathogens used in the antimicrobial screening												
	<i>Escherichia coli</i>	<i>Enterobacter aerogens</i>	<i>Bacillus subtilis</i>	<i>Klebsiella pneumoniae</i>	<i>Shigella flexneri</i>	<i>Salmonella enterica</i> ser. Typhi	<i>Staphylococcus aureus</i>	MRSA <sup>b</sup>	<i>Pseudomonas aeruginosa</i>	<i>Candida albicans</i>	<i>Microsporium gypsum</i>	<i>Microsporium canis</i>	
<i>Penicillium</i> sp. HYMS16	+	+	++	++	+	+	+	+	+	+	-	-	
<i>Phomopsis</i> sp. HYMS25	-	-	-	-	-	-	-	-	-	-	-	-	
<i>Colletotrichum gloeosporoides</i> HYMS26	-	-	+	-	-	-	-	-	-	-	-	-	
<i>Lasiodiplodia theobromae</i> HYMS42	++	+++	+++	+++	+++	+++	+++	+	++	+++	++	++	
<i>Chaetomium globosum</i> HYML 55	+++	+++	-	+++	++	+	-	-	+	-	-	-	
<i>Aspergillus</i> sp. HYML56	+	-	+	+	+	+	-	-	+	+	+	+	
<i>Pestalotiopsis mangiferae</i> HYMS64	-	-	+	+	+	+	-	-	+	+	+	+	
<i>P. mangiferae</i> HYMS65	-	-	+	+	+	+	-	-	+	+	+	+	
<i>P. mangiferae</i> HYMS66	-	-	+	+	-	+	-	-	+	+	+	+	
<i>Pestalotiopsis</i> sp. HYMS93	+++	+	+++	+	-	+	+	-	+	+	+	+	
<i>Curvularia pallescens</i> HYMS94	+	+	-	-	-	+	-	-	-	-	-	-	
<i>Colletotrichum gloeosporoides</i> HYJE10	+	+	++	++	-	++	+	-	+	-	-	-	
<i>Penicillium</i> sp. HYJE16	+	+	+++	++	++	++	-	-	+	-	-	-	
<i>Fusarium verticilloides</i> HYJE28	-	-	+	+	+	+	+	-	+	+	+	+	
Sterile morphospecies HYJE39	-	-	+	+	+	+	+	-	-	-	-	-	
Sterile morphospecies HYJE43	-	-	-	-	+	+	+	-	-	-	-	-	
Sterile morphospecies HYJE44	-	-	+	-	+	++	+	-	-	-	-	-	
<i>Trichoderma</i> sp. HYJE66	-	-	+	-	-	+	-	-	+	+	+	+	
<i>Fusarium</i> sp. HYJE70	++	++	++	++	++	++	+	-	+	+	+	+	

<sup>a</sup> + Diameter of inhibition zone <15 mm, ++ zone of inhibition 15–20 mm, +++ zone of inhibition > 20 mm, - no antimicrobial activity

<sup>b</sup> Methicillin resistant *S. aureus*

<sup>c</sup> Not done



**Fig. 3** Dose-dependent DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity of endophytic isolates from **a** *N. foetida*, **b** *H. mysorensense* and **c** *H. japonicum*

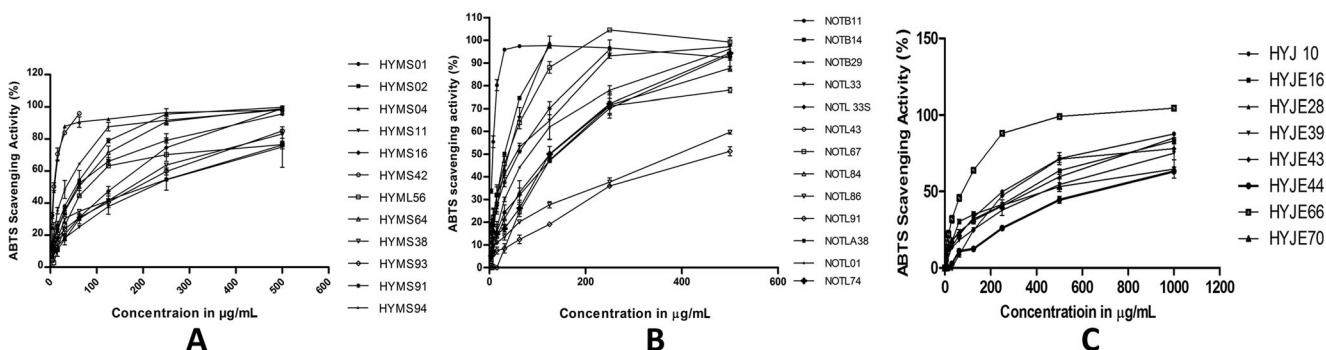
Nevertheless, the endophytic composition of plants inhabiting diverse niches has effectively not been studied because of the limitations of study models. The number of endophytes isolated from a plant depends on the number of tissue segments used, the size of the segments, processing time and the inherent growth capacity of the isolates. Fast-growing fungal genera generally mask the growth of slow-growing hyphae in the tissue, because of which most reports on endophytic isolation describe only fast-growing fungal genera. Non-culturable fungi are another limitation of endophytic studies as they do not grow under in vitro conditions despite being present in vivo. Nevertheless, culturing of surface-sterilized plant tissue segments on agar media is the most accepted method and is widely employed by researchers for the isolation of endophytes (Hyde and Soyong 2008).

This study represents the first report on the diversity and bioactivity of endophytic fungi from *H. mysorensense*, *H. japonicum* and *N. foetida* from Western Ghats forest. A total of 298 isolates were obtained from 378 tissue segments of these three medicinal plants. Identification of fungi by microscopic analysis using standard mycological manuals is a traditional and reliable approach, but identification by ITS rDNA sequencing has also been employed successfully in fungal taxonomy and phylogenetic studies. Hence, both methods were employed in the present study for the identification of endophytic isolates. The taxonomic identification of dark septate endophytes (DSE) is not yet clearly understood because of the lack of clearly defined sexual stages, absence of asexual

spores and lack of identifiable morphological traits (Mandyam and Jumpponen 2014). Similarly, sterile mycelia also could not be identified by morphological methods and could be classified only after sequence analysis. However, the lack of a comprehensive rDNA database for sterile fungi makes identification difficult even with molecular approaches. Therefore, sterile mycelia and DSEs were collected in an uncategorized group.

Identifiable endophytic biota isolated from different tissues of *N. foetida*, *H. mysorensense* and *H. japonicum* belonged to Pezizomycetes, Sordariomycetes, Dothideomycetes, and Eurotiomycetes, which are considered the major four species-rich classes of Ascomycota. Since this is the first report on the endophytes of *H. japonicum* and *H. mysorensense*, there are no other reports with which to compare it. However, earlier studies on endophytic fungal diversity associated with medicinal plants have documented the endophytic occurrence of Coelomycetes, Basidiomycetes, Hyphomycetes and Ascomycetes (Jin et al. 2013). Kurose et al. (2012) reported endophytic fungi from the Japanese weed *Fallopia japonica* that belonged only to Ascomycota.

Colonization frequency was higher for Sordariomycete members followed by the uncategorized fungal isolates, whereas Pezizomycetes and Eurotiomycetes were observed at lower frequencies (Fig. 1). U'Ren et al. (2012) also reported a similar composition of endophytes on the continental scale with decreasing values of CF in the order Sordariomycetes > Pezizomycetes > Dothidiomycetes > Leotiomycetes > Eurotiomycetes. Foliar



**Fig. 4** Dose dependent ABTS free radical scavenging activity of endophytic isolates from **a** *N. foetida*, **b** *H. mysorensense* and **c** *H. japonicum*

endophytes from the medicinal tree *Trichilia elegans* were also reported to belong to the same four classes of Ascomycota (Rhoden et al. 2012).

A total of 31 genera of endophytic fungi was isolated during the study, excluding the unidentified sterile mycelia and DSE. Some of the dominant genera identified in the present study, such as *Alternaria* spp., *Colletotrichum* spp., *Cladosporium* spp., *Chaetomium globosum*, *Curvularia* spp., *Gliocladium* spp., *Pestalotiopsis* spp., *Phoma* spp., *Phomopsis* spp., *Penicillium* spp., *Aspergillus* spp., *Nigrospora* spp., *Trichoderma* spp., *Fusarium* spp., and *Acremonium* spp. have been isolated predominantly as endophytes from numerous medicinal plants (Gautam 2014; Takemoto et al. 2014; Verma et al. 2013). Other genera such as *Botryosphaeria* sp., *Bipolaris* sp., *Cylindrocarpon* sp., *Cylindrotrichum* sp., *Lasiodiplodia* sp., *Monodictys* sp., and *Myrothecium* sp. were also reported for their endophytic occurrence in medicinal plants, though not as often as the previously mentioned dominant genera (Gao et al. 2014; Sultan et al. 2014; Yang et al. 2014). *Gliocladium* spp., isolated as endophytes from *Eucryphia cordifolia*, were reported to produce low molecular weight volatile hydrocarbons (Strobel et al. 2008). *Vermiculariopsiella* spp., and *Peyronellaea* sp. are rarely reported as endophytes. However, *Vermiculariopsiella pteridis*—a novel strain—was isolated as an endophyte from a pteridophyte *Pteris vittata* collected from Western Ghats (Dhargalkar and Bhat 2009), and *Peyronellaea* sp. was isolated as an endophyte from maize growing in a heavy metal contaminated area of China (Shen et al. 2013).

This study is the first of its kind in *H. mysorensis* and *H. japonicum*. However, Musavi and Balakrishnan (2013) reported the endophytic diversity of *N. foetida*. They reported 170 isolates from leaf, stem, seed and fruits of *N. foetida* comprising predominantly Hyphomycete members (CF = 75.29 %) with *Fusarium*, *Penicillium*, *Aspergillus*, and *Colletotrichum* as the dominant genera. Apart from these, random studies on the endophytes of *N. foetida* with the objective of isolating bioactive strains reported a few other genera, such as *Entrophosphora infrequens* (Amna et al. 2006) and *Nodulisporium* sp. (Dai et al. 2006), which have the ability to produce camptothecin.

Endophytic biota of stems and leaves of *N. foetida*, *H. mysorensis* and *H. japonicum* were assessed individually using various statistical indices. Species richness and evenness are the basic entities of the statistical assessment of population diversity. Species richness, which reflects the actual number of species in a population, is the simplest entity of any diversity study (Jost 2006). The species richness of *H. mysorensis* stem was highest among all the tissues studied, and *N. foetida* stem was lowest with only 11 different species. The species richness of *N. foetida* leaves (33), *H. mysorensis* leaves (30) and *H. japonicum* stem (33) were comparable to each other. There are no reports on endophytic biota of *H. mysorensis* and

*H. japonicum* to compare results. However, previous work on the endophytic fungi from *N. foetida* yielded only 20 different taxa from leaves, which is less than that in the present study. Nevertheless, the 12 taxa reported from the *N. foetida* stem by Musavi and Balakrishnan (2013) is similar to the results of the present study.

Shannon–Weiner entropy (H) is the most profound and useful of all diversity indices, even though the values are not linear (Jost 2006). Therefore, a number of reports on endophytic diversity have used this statistic for the assessment of alpha diversity. If the H values are considered empirically, the *H. mysorensis* stems were most diverse, with the H value of 5.082, and *N. foetida* stems were least diverse, with an H value of 2.172. Endophytic diversity in the leaves of *N. foetida* and *H. mysorensis* and stems of *H. japonicum* are comparable to each other, with H values of 3.32, 3.305 and 3.215, respectively. The data correlates with species richness data, which is not surprising as assessments rely on the species richness, rather than on dominance. Previous work has deduced a lower H value of 2.794 and 2.362 for *N. foetida* leaves and stems, respectively (Musavi and Balakrishnan 2013).

Since the Shannon entropy is not linear, the true diversity with effective species numbers would have been preferable for comparison purposes. In the present study, the Fischer- $\alpha$  and exponent of H values have been used to arrive at true diversity (Table 3). The results correlate with the previous two diversity measures where *H. mysorensis* stem had the highest value of 33.85 and *N. foetida* stems had the lowest (15.059) effective species in it. The Simpson Dominance index (D) and Simpson Diversity index (1–D) consider the concentration of individuals in various taxa. The diversity index will have a value between 0 and 1, where ‘0’ represents no diversity and ‘1’ represents maximum diversity (Simpson 1949). High values of these indices in all the studied tissues suggest greater diversity with the absence of significant dominance of any species, which further justifies the Shannon diversity values. Evenness and equitability indices justify the higher Simpson diversity values, which suggest the even distribution of individuals among different taxa. However, endophytic fungi were reported to be distributed more evenly in *N. foetida* leaves and stem (Musavi and Balakrishnan 2013). The diversity of endophytes in different tissues of a plant was compared with the help of Sorenson’s index and Jaccard coefficient measures, where the values will be between 0 and 1, with ‘zero’ being complete dissimilarity and ‘one’ being complete similarity. The values suggest that the endophytic composition of leaves and stems of *H. mysorensis* is more than 50 % similar. But the leaves and stems of *N. foetida* differ in terms of endophytic composition as the indices are less than 50 %.

Endophytic diversity of *N. foetida* leaves, tissues from *H. mysorensis* and *H. japonicum* do not differ significantly ( $P > 0.05$ ) as deduced by the diversity t-test. However, only

*N. foetida* stems differed significantly ( $P < 0.05$ ) from other tissues in endophytic composition.

Of the three medicinal plants considered in this study, *N. foetida* is a tree, *H. mysorensis* is a shrub and *H. japonicum* is a small herb. Generally, herbaceous plants were expected to yield diverse fungal biota owing to their proximity to the rhizosphere. In the present study, all three plants were equally diverse in their endophytic composition. Since all plants were collected from a biodiversity hotspot, the endophytic biota may be equally diverse. Equal distribution of endophytes within the tissues and the absence of dominance suggest the putative nature of the endophytes. Since most of the taxa are shared among the tissues and also among the plants, these endophytes may not be tissue specific.

All 298 fungi isolated from the three medicinal plants were subjected to preliminary screening for antimicrobial activity by the agar disc diffusion method. Finally, 13.09 % of the isolates were found to have potent antimicrobial activity against at least one of the tested bacterial strains. Some earlier reports also mention such a smaller proportion of total endophytic isolates having bioactive potential (Nath et al. 2013). Endophytic fungal strains belonging to 15 genera recorded considerable antimicrobial activity. Of these, a few strains, such as *Bionectria ochroleuca* NOTL33, *Chaetomium globosum* HYML55, *Colletotrichum gloeosporoides* HYJE10, *Penicillium* sp. HYJE16, *Alternaria brassicae* HYMS01, *Pestalotiopsis* sp. HYMS02, and two *Aspergillus* spp. (HYML56 and NOTL91) were very active against the pathogenic microorganisms used in the study. A total of eight volatile antimicrobial molecules had been identified from ethyl acetate extracts of *Bionectria ochroleuca* NOTL33 (Samaga et al. 2014a) and an antimicrobial cytochalasan had been characterized from *Chaetomium globosum* HYML55 (Samaga et al. 2014b) and already published. The antimicrobial activity of crude extracts of endophytic fungi is well recorded in the literature (Sultan et al. 2014; Yang et al. 2014), but no reports are available on the antimicrobial activity of endophytes from *H. japonicum* and *H. mysorensis*.

The free radical scavenging activity of endophytic extracts could be attributed to the presence of phenolic compounds (Huang et al. 2008). Formation of singlet reactive oxygen species is a common but crucial phenomenon in plants during photorespiration in combination with abiotic stress. These reactive oxygen species (ROS) could be fatal for plants if the equilibrium between ROS production and antioxidant scavenging is perturbed (Foyer and Shigeoka 2011). Mutualistic endophytes help their hosts to recover from ROS-mediated effects by producing antioxidant secondary metabolites. This was evident by previous reports that endophytic assemblages confer drought resistance capacity on their hosts. Notable antioxidant activity in the endophytes substantiates the statement that antioxidant activity could be a currency in the mutualism of endophytes (Hamilton and Bauerle 2012).

This study is based on data from a single season. Hence, a comparative study of seasonal variation of endophyte composition in these plants could help clarify our comprehension of environmental influences on plant–endophyte interactions. The seasonal occurrence of novel endophytic strains with novel biosynthetic capacity may be useful for their industrial and agricultural exploitation.

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