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ORIGINAL ARTICLE

Bionectria ochroleuca NOTL33—an endophytic fungus from *Nothapodytes foetida* producing antimicrobial and free radical scavenging metabolites

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Abstract Endophytic fungi are reported to produce diverse classes of secondary metabolites. This study investigated the antimicrobial and free radical scavenging activity of a foliar endophytic fungus from *Nothapodytes foetida*, a medium sized tree known to produce the antineoplastic compound camptothecin. The fungal isolate was identified as *Bionectria ochroleuca* based on the ITS rDNA analysis. The differences among endophytic, pathogenic and free living *Bionectria ochroleuca* were established by RNA secondary structure analysis. The metabolites showed a broad spectrum of antibacterial, antifungal and anti-dermatophytic activity. Minimum inhibitory concentration values of ethyl acetate extracts were in the range of 78–625 μg/mL against all test organisms, except for *Pseudomonas aeruginosa* (5 mg/mL). Antimicrobial components in the ethyl acetate extract were identified by GC-MS analysis. The isolate was also produced volatile antifungal compounds. A dose-dependent free radical quenching was observed in the ethyl acetate extract. This is the first report on *Bionectria* sp. as an endophyte of *N. foetida*. The results indicate that the *B. ochroleuca* NOTL33 isolate is a potential source of antimicrobial agents and could be used as an effective biofumigant.

Keywords Endophyte · *Nothapodytes* · *Bionectria* · Free radical scavenging · Volatile antimicrobials

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Introduction

Microorganisms that spend the whole or part of their life cycle colonising, inter- or intra-cellularly, the healthy tissues of host plants, typically causing no apparent symptoms of disease, are called endophytes (Zhao et al. 2010). The endophytic fungi represent unique biotopes and are phylogenetically distinct from their free-living counterparts (Syed et al. 2009). Since the metabolites that microorganisms produce seem to be characteristic of certain biotopes, endophytes are currently considered to be a wellspring of novel secondary metabolites offering the potential for medical, agricultural and industrial exploitation (Strobel and Daisy 2003). Myriad metabolites belonging to different chemical categories, such as alkaloids, steroids, terpenoids, isocoumarin derivatives, quinones, flavonoids, cytochalasines, furandiones, phenylpropanoids, lignans, peptides, phenol, phenolic acids, aliphatic compounds and chlorinated metabolites, have been reported from endophytes, which are known for their antibiotic, antiviral, volatile antibiotic, anticancer, antioxidant, insecticidal, antidiabetic and immunosuppressive properties (Verma et al. 2009). Plants with medicinal value or those growing in biodiversity hotspots have been hypothesised to harbour hyperdiverse endophytic biota with useful bioactive compounds (Strobel and Daisy 2003).

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, 9-methoxy-20-O-acetylcamptothecin (Govindachari and Viswanathan 1972; Srinivas and Das 2003), which exhibit antimicrobial, antiviral and anticancer properties (Kumar et al. 2002; Liu et al. 2010). Considering the facts that the tree has medicinal value and that it grows in an area distinguished as one among the world's ten most

important biodiversity hotspots (Myers et al. 2000), the present study was aimed at the isolation of endophytic fungi from *N. foetida*, assessment of antimicrobial and free radical scavenging activity of secondary metabolites from endophytic isolates, and compositional analysis of crude extracts showing significant bioactivity.

Materials and methods

Isolation of endophytic fungi

Healthy leaves of *N. foetida* were collected from a full grown tree near the Aloor region of Hassan, Karnataka, India (12.94501°N, 75.89184°E). Plant materials were identified taxonomically and the herbarium maintained at the Department of Studies in Microbiology, University of Mysore (Voucher number: MGMB/214/2009). Endophytic fungi were isolated using a method described previously by Cannon and Simmons (2002) with modifications. Samples were washed with running tap water and surface sterilised by sequential treatments with 0.1 % mercuric chloride for 1 min and 90 % ethanol for 2 min followed by a sterile water wash. Leaf pieces of 0.5 cm^2 were placed onto sterile water agar (pH 4.8, 1.2 % agar) and incubated at ambient temperature in the dark until hyphae emerged from the cut ends. The cultures were maintained on Potato dextrose agar (HiMedia, Mumbai, India) slants at 4 °C for further studies.

Identification of endophytic isolates

The endophytic fungal isolates were identified up to genus level based on the morphological features like colony morphology, pigmentation, growth pattern, spore structures and other hyphal characteristics with the help of the standard mycological manuals (Ellis 1971, 1976; Gilman 1971).

Identification of *B. ochroleuca* by DNA sequence analysis of the internally transcribed spacer region

Genomic DNA was extracted from the lyophilized fungal mat of isolate NOTL33 by the cetyltrimethylammonium bromide (CTAB) method. The nuclear ribosomal DNA and internally transcribed spacer (ITS) region were amplified using ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATG-3′) primers (White et al. 1990). The reaction volume was 25 μL, comprising of 12.5 μL 2× PCR master mix (Genei, Bangalore, India), 1 μL each of forward and reverse primers (10 pmol/μL) and template DNA (120 ng), 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 amplicon was sequenced using ITS1 and ITS4 primers in two different sequencing reactions. A contiguous sequence out of two sequences was generated using CAP3 sequence assembly program (Huang and Madan 1999) and submitted to the GenBank nucleotide collection.

Taxon sampling and phylogenetic analysis

Sequences with high similarity to the sequence of the isolate were retrieved from the GenBank database using the megablast program. The 40 closest hits out of 100 sequences displayed on the search were downloaded, of which 33 sequences with a complete stretch comprising ITS1, 5.8S, and ITS2 were selected for phylogenetic analysis (Lv et al. 2010). Flanking partial 18S and 23S rRNA regions were cropped so that each sequence contains a complete stretch of ITS1, 5.8S rDNA and ITS2. Sequences were aligned using the ClustalW program utilizing the default settings and the tree was generated using MEGA 5 (Tamura et al. 2011) by the maximum parsimony (MP) method. A bootstrap consensus tree was inferred from 1,000 replicates (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 5 % bootstrap replicates were collapsed. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar 2000) with search level 1, in which the initial trees were obtained with the random addition of sequences (ten replicates).

RNA secondary structure analysis

A total of eight rDNA sequences (each comprising of ITS1, 5.8S rDNA and ITS2) from the organisms sharing the same clade of the *Bionectria ochroleuca* NOTL33 were selected to generate the RNA secondary structure using the mfold web server (Zuker 2003) with a pre-set temperature of 37 °C and the following default conditions: 1 M NaCl with no divalent ions, maximum number of nucleotides in a bulge or loop limited to 30; maximum asymmetry of an interior/bulge loop 30; percentage suboptimality number 5; upper bound on number of computed folding 50. Out of various similar structures obtained, the structures with the highest negative free energy were selected for the comparison.

Fermentation and extraction

Wide mouth bottles of 200 mL capacity were filled with 45 mL fermentation medium [Potato dextrose broth (HiMedia) 20 g/L, Soytone (HiMedia) 5 g/L] and inoculated with three discs (6 mm) of actively growing hyphae. The flasks were kept undisturbed for 20 days at ambient (28 °C) temperature.

After incubation, the mycelia were separated from the fermentation broth by filtering through two layers of destarched

cheese cloth, and finely ground in a mixer grinder. The filtrate and the mycelial slurry were separately extracted three times with an equal volume of ethyl acetate (SD Fine chemicals, Mumbai, India). The fractions were pooled together, demoistured with sodium sulphate and the solvent evaporated under vacuum using a rotary evaporator. The residue was dissolved in minimal amount of ethyl acetate and stored at −20 °C until tested further.

Evaluation of antimicrobial activity

The microbial strains used in the study were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India and Department of Studies in Microbiology, University of Mysore, Karnataka, India. The strains used were *Pseudomonas aeruginosa* (MTCC 7093), *Escherichia coli* (MTCC 40), *Enterobacter aerogenes* (MTCC 111), *Klebsiella pneumoniae* (MTCC 661), *Shigella flexneri* (MTCC 1457), *Bacillus subtilis* (MTCC 121), *Salmonella enterica* ser. Typhi (MTCC 733), *Staphylococcus aureus* (MTCC 7443), Methicillin resistant *Staphylococcus aureus* (Clinical isolate), *Candida albicans* (MTCC 183), *Microsporum gypseum* (MTCC 2830), *Microsporum canis* (MTCC 2831), *Aspergillus nidulans* (MTCC 803), *Aspergillus terreus* (MTCC 2580), *Aspergillus flaviceps* (MTCC 1990), *Aspergillus fumigatus* (MTCC 3008), *Aspergillus clavatus* (MTCC 1323), *Aspergillus parasiticus* (MTCC 2797) and *Penicillium citrinum* (MTCC 1784). Bacteria, yeasts and filamentous fungi were maintained on nutrient agar, Sabouraud's dextrose agar and potato dextrose agar slants, respectively, at 4 °C with routine subculturing.

The antimicrobial assay was performed using the paper disc diffusion method (Bauer et al. 1966). The crude extracts were tested at 400 μg per disc concentrations. Discs loaded with solvent alone (40 μL) were used as the negative control. Chloramphenicol (HiMedia, 30 μg per disc) and Nystatin discs were used as positive controls for bacteria and fungi, respectively. The inhibition zones around the discs were measured using zone scale (HiMedia, India), and the mean of three replicate readings were recorded.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined by the broth micro-dilution method (Eloff 1998). The test was carried out in a 96-well microtitre plate (Tarsons, Kolkata, India). The total reaction mixture of 200 μL consisted of 180 μL Mueller Hinton Broth (HiMedia), 10 μL extract in methanol and 10 μL standardised inoculum. The extracts were tested in the range from 5 mg/mL to 0.0195 mg/mL of the reaction volume. After incubation, microbial growth in the wells was visualised by using triphenyl tetrazolium chloride (TTC) as an indicator. The

Table 1 Screening of foliar endophytes from *Nothapodytes foetida* for antibacterial activity[a]

Test organisms	Antimicrobial activity of foliar endophytic isolates from *N. foetida* (isolates coded as NOTL)													
	NOTL 11	NOTL 12	NOTL 26	NOTL 33	NOTL 37	NOTL 42	NOTL 43	NOTL 46	NOTL 72	NOTL 74	NOTL 79	NOTL 91	NOTL 92	
Escherichia coli (MTCC 40)	+	+	−	+++	+++	−	+	−	+	−	++	+++	−	
Enterobacter aerogens (MTCC 111)	+	+	+	+++	+++	+	+	−	−	−	+++	++	−	
Bacillus subtilis (MTCC 121)	++	−	−	+++	−	−	−	−	+	+	−	++	−	
Klebsiella pneumoniae (MTCC 661)	−	−	−	+++	−	−	−	−	−	−	+++	+++	−	
Shigella flexneri (MTCC 1457)	++	−	−	+++	−	−	−	−	++	+	++	+++	−	
Salmonella enterica ser. Typhi (MTCC 733)	+	−	−	+++	++	−	−	−	+	−	++	+++	++	
Staphylococcus aureus (MTCC 7443)	−	−	−	+	+++	−	−	−	ND	−	+++	−	+	
Pseudomonas aeruginosa (MTCC 7083)	−	+	−	−	−	+	−	++	++	−	+++	+++	+	
Methycillin resistant *Staphylococcus aureus*	−	−	++	++	+++	−	−	−	ND	−	+++	−	−	

[a] − No antimicrobial activity, + diameter of the inhibition zone < 15 mm, ++ zone of inhibition from 15 mm to 20 mm, +++ The zone of inhibition > 20 mm, *ND* not done

assay was carried out in triplicate. Chloramphenicol and nystatin were used as positive controls at similar dilutions against bacteria and dermatophytes, respectively. Broth inoculated with methanol only served as a negative control (growth controls) and the un-inoculated broth was maintained as a sterility control. The least concentration with no visible growth was considered as the MIC. Broth from wells containing the MIC concentration and above was streaked onto agar medium to check cell viability. The least concentration with no viable cells was recorded as the minimum microbicidal concentration (MMC).

Test for volatile antimicrobials

The production of volatile antimicrobials was tested as described previously (Ting et al. 2011) with modifications. The *B. ochroleuca* NOTL33 isolate was grown for 5 days on PDA plates as a single colony in each plate. The test fungal strains were point-inoculated onto PDA plates. The lids of the plates were removed and the test fungi plates were inverted over the pre-incubated endophytic plates and sealed airtight with plastic wrap. The point inoculated test fungi alone served as the control. The percentage inhibition of growth of test fungi over the control was calculated using the formula,

$$\text{Growth inhibition } (\%) = \left[\left(D_{control} - D_{test}\right) \Big/ D_{control}\right] \times 100$$

where D = colony diameter. The experiment was repeated three times and the result expressed as mean inhibition.

DPPH radical scavenging activity

Free radical scavenging activity of the ethyl acetate extract was determined as described previously (Ho et al. 2012) with modifications. The two-fold dilution of extracts in the range from 5 mg/mL to 0.039 mg/mL in methanol (100 μL) were mixed with 100 μL DPPH solution (1,1-diphenyl-2-picrylhydrazyl, Sigma, Sternheim, Germany, 40 μmol/L). A control was maintained by adding 100 μL DPPH to 100 μL 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 following formula:

$$\text{DPPH scavenging activity } (\%) = \left[\left(A_{control} - A_{sample}\right) \Big/ A_{control}\right] \times 100$$

The data are presented as the means of triplicate experiments. Antioxidant activity was expressed as milligram equivalents of ascorbic acid (AA).

ABTS radical scavenging activity

The free radical scavenging activity of the crude extracts was determined using 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) stable cationic free radicals (Osman et al. 2011). 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. Various concentrations of the extract in 10 μL aliquots (5 mg/mL to 0.039 mg/mL in two-fold dilutions in methanol) was mixed with 200 μL standardized ABTS solution (absorbance adjusted to 0.6±0.05 at 734 nm) and the absorbance was read at 734 nm every 5 min up to 60 min. The ABTS quenching activity was calculated by the formula,

$$\text{ABTS scavenging activity}(\%) = \left[\left(A_{control} - A_{sample}\right) \Big/ A_{control}\right] \times 100$$

The data are presented as the means of triplicate experiments. The concentration required for 50 % reduction of ABTS radical (IC_{50}) was determined graphically. The antioxidant capacity was expressed as milligram equivalents of AA.

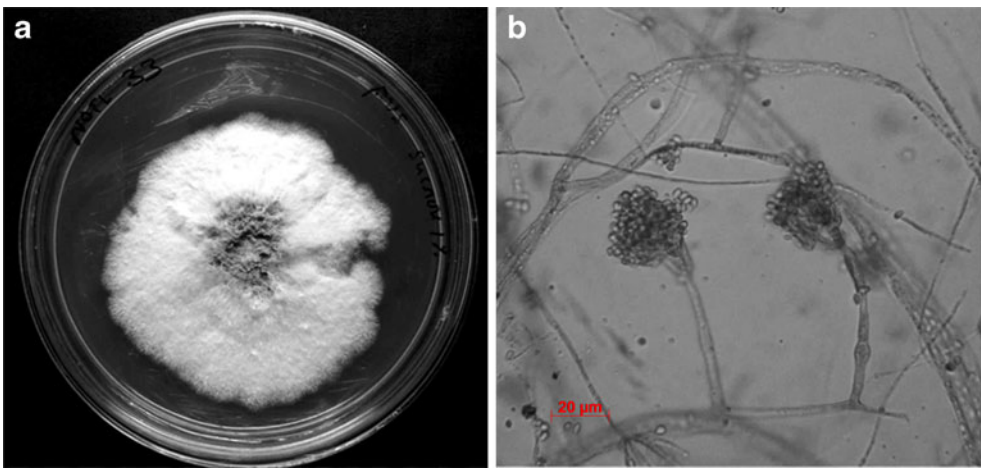

Fig. 1 Colony morphology (**a**) and microscopic features at 40× magnification (**b**) of the endophytic *Bionectria ochroleuca* NOTL33 isolated from *Nothapodytes foetida*

GC-MS analysis

The composition of *B. ochroleuca* NOTL33 ethyl acetate extract was analysed using a Shimadzu table top GC-MS (GC 17A ver. 3) equipped with a DB-1 capillary column (30 m, ID = 0.25 mm, film thickness = 0.15 mm). The conditions during the analysis were as follows: initial injection temperature: 250 °C, interface temperature: 230 °C and the oven temperature was raised from 60 °C to 250 °C over a period of 5 min to 30 min. The column flow of nitrogen carrier

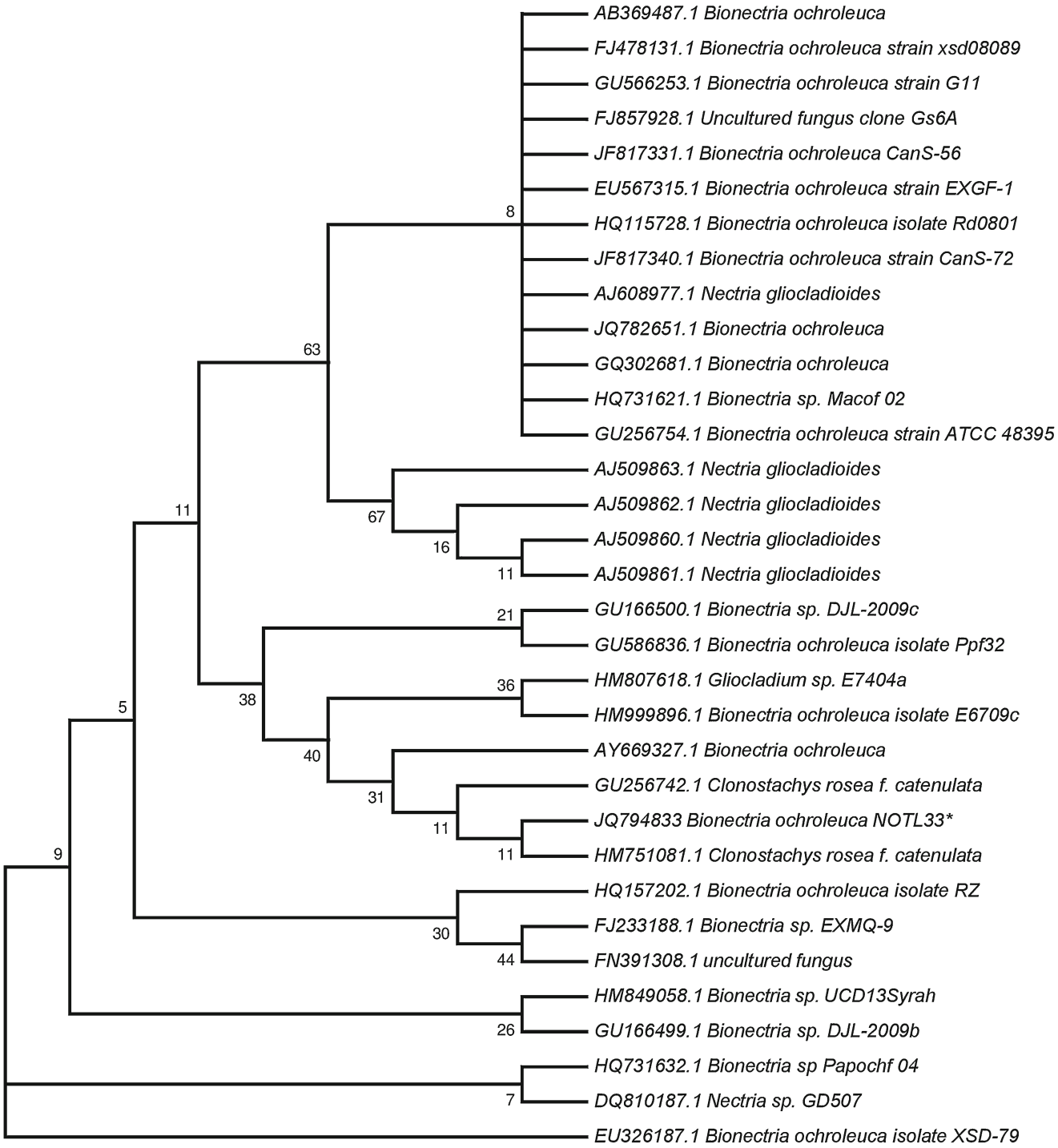

Fig. 2 Phylogenetic tree showing the evolutionary relationship of endophytic *B. ochroleuca* NOTL33 (JQ794833.1) with its closest BLAST hits. Evolutionary history was inferred using the maximum parsimony method tested with 1,000 boot strappings. The analysis involved 33 nucleotide sequences and 481 positions in the final dataset. All positions with less than 0 % site coverage were eliminated. The evolutionary relationships were inferred using MEGA-5 software

Table 2 Organisms clustered in the clade of *Bionectria ochroleuca* NOTL33 isolate with their lifestyle, source and GenBank accession numbers

Organism	Source	Habit	Accession no.
Clonostachys rosea f. *catenulata*	Unknown	ATCC	GU256742.1
Bionectria ochroleuca	*Pleurotus eryngii*	Pathogen	AY669327.1
Bionectria ochroleuca NOTL33	*Nothapodytes foetida*	Endophyte	JQ794833.1[a]
Clonostachys rosea f. *catenulata*	*Aspergillus flavus* sclerotia	Mycoparasite	HM751081.1
Bionectria ochroleuca isolate E6709c	Rainforest plants	Endophyte	HM999896.1
Gliocladium sp. E7404a	Rainforest plants	Endophyte	HM807618.1
Bionectria ochroleuca isolate Ppf32	*Paris polyphylla*	Endophyte	GU586836.1
Bionectria sp. DJL-2009c	*Changnienia amoena* Chien	Endophyte	GU166500.1

[a]Accession number of the fungus under study

gas was set at linear velocity of 45.9 cm/s with a flow rate of 1.3 mL/min (total flow 38.9 mL/min). Mass spectrometer (GCMS-QP5050A) detector with scan acquisition mode was set at m/z from 40 to 500 with scan speed of 1,000 over a 30-min time period, interface temperature 230 °C and detector gain 1.3 kV. The mass peaks were interpreted tentatively based on the mass peak split pattern in the mass spectrum.

Data analysis

Antimicrobial activity was expressed as the mean value ± standard deviation of triplicate readings. The activity of the extract was compared to that of standard antibiotic by a two-tailed paired *t*-test assuming "no difference in the mean antimicrobial activity between the extract and the antibiotic"

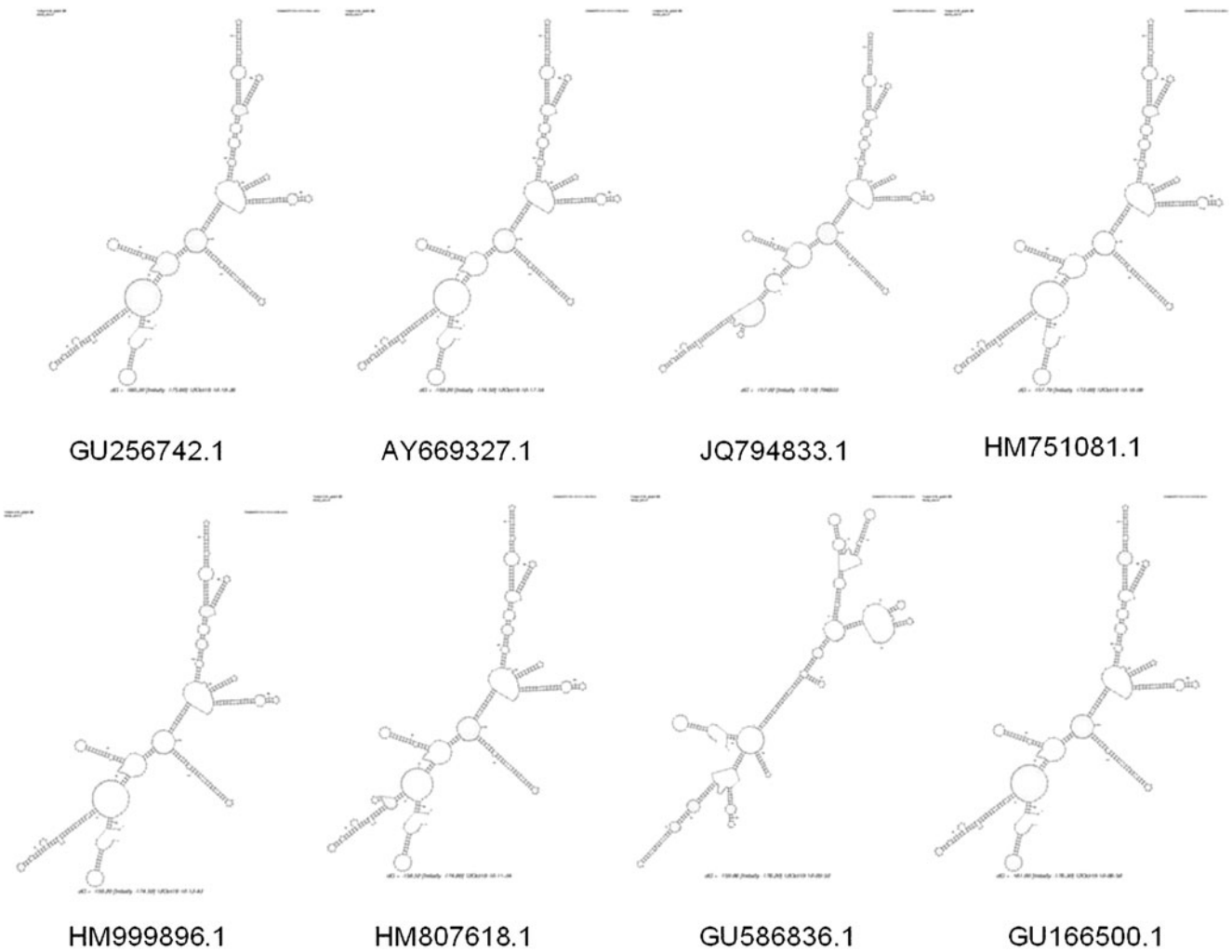

Fig. 3 Secondary structures of rRNA (complete stretch of ITS1–5.8S–ITS2 sequences) of eight organisms clustered in the same clade as the isolate *B. ochroleuca* NOTL33

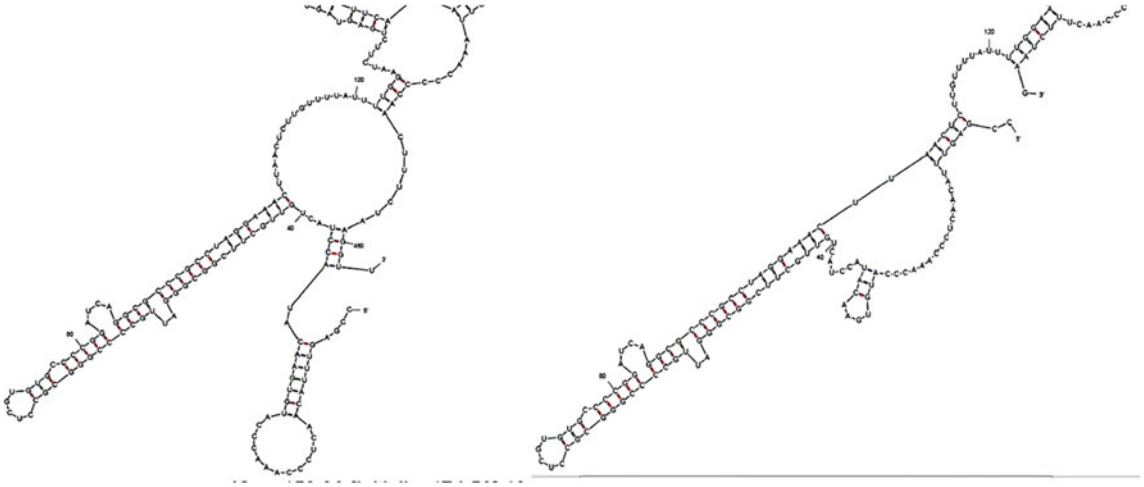

Fig. 4 Unique secondary structure of *B. ochroleuca* NOTL33 (*right*), which differs from that of its homologous pathogenic counterpart AY669327.1 (*left*)

as the null hypothesis. The significance of the inhibition of test fungi by volatile antimicrobials of the isolate over the control was calculated by a one-tailed paired *t*-test. The correlation between DPPH and ABTS scavenging activity of the extract was assessed by linear regression analysis. Statistical analysis was performed using the Microsoft Excel spreadsheet application.

Results and discussion

Leaf discs of *Nothapodytes foetida* harbored a total of 74 isolates belonging to the genera *Bionectria*, *Gliocladium*, *Phomopsis*, *Xylaria*, *Aspergillus*, *Alternaria*, *Penicillium*, *Phoma*, *Cylindrotrichum*, *Monodictys*, *Chaetomium*, *Helminthosporium*, *Pestalotiopsis*, *Fusarium*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Botryosphaerea*, *Mycelia sterilia* and other unidentified genera. The isolation frequency of the foliar endophytic fungi was 79.56 %. The endophytic association is ubiquitous in nature and was found in every plant species examined (Guo et al. 2008). Plants with medicinal value or those growing in regions of great biodiversity are of particular interest as a source to isolate endophytes as they harbour hyperdiverse endophytic biota with the potential for production of novel bioactive molecules (Strobel and Daisy 2003). This fact was the rationale behind the selection of *Nothapodytes foetida* from Western Ghats.

The endophytic isolates were fermented for 20 days. Fermented broths and blended fungal mats were extracted separately with ethyl acetate. The ethyl acetate extracts were screened for antimicrobial activity and a few isolates showed considerable antimicrobial activity against at least some of the test organisms used (Table 1). An endophytic isolate coded as 'NOTL33' was selected for further characterisation based on the results of preliminary screening. The morphological features of isolate NOTL33 are shown in Fig. 1. The rDNA sequence of this isolate (NCBI accession no. JQ794833.1) shared 99 % homology with *Bionectria ochroleuca* on BLAST analysis. This study is the first report of the endophytic occurrence of *Bionectria ochroleuca* in *Nothapodytes foetida*. The isolate was considered as an 'endophyte' based on the definition provided by Hallmann et al. (1997); however, the endophytic occurrence of *Bionectria* spp. has been reported previously by several

Table 3 Antibacterial and antidermatophytic activity of ethyl acetate extracts of endophytic *B. ochroleuca* NOTL33 strain

Tested microorganisms	Diameter of inhibition zone[a] (in mm)
Escherichia coli (MTCC 40)	31.5±0.70
Enterobacter aerogens (MTCC 111)	21.0±2.82
Bacillus subtilis (MTCC 121)	30.5±0.70
Klebsiella pneumoniae (MTCC 661)	25.0±0.00
Shigella flexneri (MTCC 1457)	34.0±0.00
Salmonella enterica ser. Typhi (MTCC 733)	23.34±1.15
Staphylococcus aureus (MTCC 7443)	15.34±0.57
Pseudomonas aeruginosa (MTCC 7083)	NA[b]
Methycillin resistant *Staphylococcus aureus*	19.0±0.00
Aspergillus nidulans (MTCC 803)	25.5±0.70
Aspergillus terreus (MTCC 2580)	25.0±0.00
Aspergillus flaviceps (MTCC 1990)	39.0±1.41
Aspergillus fumigatus (MTCC 3008)	19.5±0.70
Aspergillus clavatus (MTCC 1323)	39.5±0.70
Penicillium citrinum (MTCC 1784)	33.0±0.00
Aspergillus parasiticus (MTCC 2797)	20.0±0.00

[a] Mean value ± SD (in mm) of triplicate values, including the diameter of the disc (6 mm)

[b] No activity

Table 4 Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) of ethyl acetate extracts of *B. ochroleuca* NOTL33

Test organism	MIC in mg/mL	MMC in mg/mL
Shigella flexneri (MTCC 1457)	0.078125	0.15625
Salmonella enterica ser. Typhi (MTCC 733)	0.31250	0.625
Staphylococcus aureus (MTCC 7443)	0.15625	0.3125
Pseudomonas aeruginosa (MTCC 7083)	5.0	–
Candida albicans (MTCC 183)	0.15625	0.3125
Microsporum canis (MTCC 2831)	0.31250	0.625
Microsporum gypseum (MTCC 2830)	0.6250	0.625

workers: Chen et al. (2011) and Ebrahim et al. (2012) isolated *Bionectria ochroleuca* as a foliar endophyte from *Dendrobium* sp. and *Sonneratia caseolaris*, respectively.

The fungus *B. ochroleuca* has been isolated as endophyte, pathogen and free living forms. The endophytes can remain inside the host as latent pathogens, which may change over time depending on various factors such as environmental stress, plant senescence and the efficacy of the plant defence response (Schulz and Boyle 2005). The horizontal transmission of endophytes from environment into the host is also common phenomenon, which marks their putative association. Most foliar endophytes, which grow between the cells of the leaves, are of this category (Syed et al. 2009). Therefore, a phylogenetic tree (Fig. 2) was generated taking the rDNA sequences of closest BLAST hits of the query sequence, to test the clustering of our isolate among endophytes, pathogens and environmental isolates. The isolate was clustered with a mycoparasitic isolate *Clonostachys rosea* f. *catenulata* (HM751081.1). The whole super-clade comprised a total of eight organisms, of which six were endophytes, one an ATCC culture of unknown origin and one a pathogen (Table 2). The isolate was more homologous to the mycoparasitic and pathogenic strains, although not supported by significant bootstrapping. Thus, the phylogenetic analysis indicated that the *Bionectria ochroleuca* NOTL33 isolate is distinct from its closest BLAST hits. This fact was further substantiated by RNA secondary structure analysis; structures are more conserved than sequences. The ITS sequence has been used as a possible marker in molecular systematics and phylogenetic reconstruction. A mismatch in two related secondary structures is defined as conserved if it does not cause a stem/loop transition. In contrast, a nonconserved mismatch is one that altered base-pairing and converted a loop to a stem or a stem to a loop (Padhi and Tayung 2012; Pei et al. 2012). The RNA secondary structures of all eight organisms in the clade were analysed. The secondary structure of *B. ochroleca* NOTL33 was evidently distinct from that of pathogenic and other endophytic isolates (Fig. 3). The stem and loop structure near the third base at the 5′- end was missing in the *B. ochroleuca* NOTL33 sequence, which makes it distinct and unique among other members of the clade (Fig. 4). Therefore, this observation proves that endophytes represent unique biotopes and are phylogenetically distinct from their free-living counterparts (Syed et al. 2009). Hence, the isolate represents a unique biotope that could be expected to possess better bioactivity and the capacity to produce unique metabolites.

Ethyl acetate extracts from *B. ochroleuca* NOTL33 were both antibacterial and antifungal (Table 3). The insignificant *t*-statistics (P=0.380579) confirmed the null hypothesis, which suggests that the antibacterial activity of the extract was comparable to that of chloramphenicol. The MIC and MMC of the extracts is given in Table 4. The MIC of the extracts was in the range of 78–312 μg/mL for the tested organisms except for *P. aeruginosa*, against which the MIC was 5 mg/mL. The MIC and minimum bactericidal concentration of the extract were lower than those of the reference antibiotic streptomycin. The extract was bacteriostatic in nature against *P. aeruginosa* and bactericidal against other test strains used. Shan et al. (2012a, b) reported similarly weak antimicrobial activity against *P. aeruginosa* in extracts of endophytic *Bionectria ochroleuca*, isolated from *Macleaya cordata* and *Paris polyphylla* var. *chinensis*, However, the distinction of molecular identity of *Bionectria ochroleuca* NOTL33 strain from the above two bioactive endophytic *Bionectria ochroleuca* (GenBank

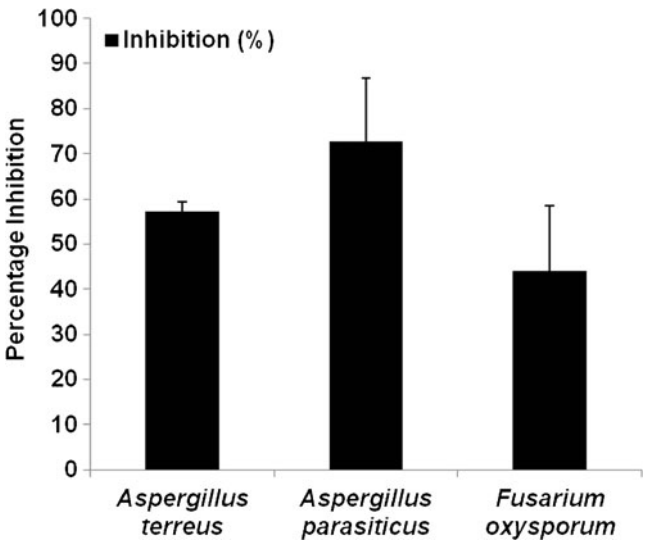

Fig. 5 Effect of volatile antimycotic compounds produced by endophytic *B. ochroleuca* NOTL33 on the growth of pathogenic fungi

Table 5 Low molecular weight constituents of the endophytic *B. ochroleuca* NOTL33 ethyl acetate extract identified based on mass peak fragmentation in GC–MS analysis

Peak number	Retention time (min)	Area %	Mass	Major mass peaks	Identified molecule
1	11.972	7.66	114	71, 57, 43	Heptanone
2	15.865	2.13	96	96,95,67, 42	Furfural
3	16.597	14.77	172	115, 85,57,43	Amyl-isovalerate
4	19.765	0.38	168	168, 151, 125, 111, 97,83,69,55,41	Decanoic acid
5	22.030	18.17	110	110, 109, 95, 81	Methyl furfural
6	22.775	1.51	262	262, 261, 219, 191, 163, 135, 91, 41	Benzyl decanoate
7	23.119	16.86	256	256, 239, 211, 57, 43	Palmitic acid
8	27.646	1.19	190	190, 119, 91, 64, 57, 43, 38	Benzyl pentyl ketone

accession numbers HQ731632 and HQ731621) was evident from the dendrogram (Fig. 2). The endophytic *Bionectria ochroleuca* isolated from mangrove plants was reported to produce bioactive peptides named Pullularins E and F (Ebrahim et al. 2012), Bionectramide A-D and Verticillin D (Kjer 2012).

Since, the MIC of the *Bionectria ochroleuca* NOTL33 ethyl acetate extract was below 500 μg/mL and the isolate represents a distinct biotope, the active components from the extracts could be candidates for the development of antimicrobial drugs.

The volatile compounds produced by *Bionectria ochroleuca* NOTL33 significantly inhibited the growth of all the three plant pathogenic fungi tested (Fig. 5). The growth of *Aspergillus terreus*, *Aspergillus parasiticus* and *Fusarium oxysporum* was inhibited by 57.13 % (P=0.002618), 72.75 % (P=0.031639) and 44.03 % (P=0.008367), respectively. No previous reports are available on the volatile antimicrobial production by *B. ochroleuca*. However, the production of volatile antimicrobial compounds (Stinson et al. 2003) and small molecular weight hydrocarbons (Strobel et al. 2008) have been reported from *Gliocladium roseum* NRRL 50072, an endophyte from *Eucryphia cordifolia*—an anamorph of *B. ochroleuca* (Uniprot 2012). This strain recorded maximum growth inhibition of up to 65.3 % against *Aspergillus ochraceous*, whereas the *Fusarium oxysporum* culture was virtually resistant to the volatiles. In the present study, maximum inhibition of up to 72.75 % was recorded for *Aspergillus parasiticus* and the *Fusarium oxysporum* culture was significantly inhibited up to 44.03 % after the same period of incubation.

The ethyl acetate extract of the NOTL33 isolate was subjected to GC–MS analysis to detect antimicrobial constituents in the extract. Based on the mass peak fragmentation pattern, eight antimicrobial compounds were identified (Table 5). The antimicrobial activity of heptanone and its derivatives (Mendonça et al. 2009), furfural (Rossmoore 2001), and decanoic acid (Isaacs 2001) had been reported previously. Amyl isovalerate was reported as one of the major components of many plant essential oils with antimicrobial properties (Morris et al. 1979). Because of the diversity and complexity of the natural mixtures of bioactive compounds in the crude extracts from natural origin, it is rather difficult to characterize every compound present and elucidate its structure in a single study; however, the purification of other active components and spectral analysis is being carried out.

The free radical scavenging activity was assessed by using DPPH and ABTS cation free radicals. The radical scavenging activity of ethyl acetate extract of *B. ochroleuca* NOTL33 isolate was moderate when compared to standard antioxidants such as butylated hydroxy anisole (BHA). The extract exhibited a dose-dependent scavenging activity (Fig. 6). At 5 mg/mL concentration, NOTL33 extract quenched a maximum of 46.17 % DPPH free radicals. However, the ABTS cation radical was quenched upto 72.16 %. The total ABTS quenching activity was expressed as milligram equivalents of ascorbic acid determined by a standard graph of ascorbic acid (R^2=0.9901). The total antioxidant capacity of NOTL33 ethyl acetate extract was 18.17 mg AA/g of the extracts. A strong correlation was seen between the DPPH and ABTS quenching activity (R^2=0.99327) as determined by linear correlation analysis.

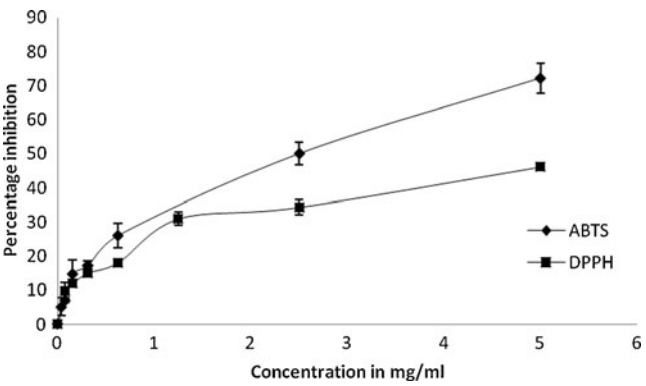

Fig. 6 Dose dependent 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of endophytic *Bionectria ochroleuca* NOTL33 ethyl acetate extract

In living organisms, oxidative stress created by reactive oxygen species (ROS) resulting from metabolism, in the form of superoxide anion (O^{-2}), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2) and nitric oxide (NO) leads to conditions like cancer, stroke, myocardial infarctions, diabetes, septic and haemorrhagic shock, and neurodegenerative diseases by inducing biomolecular oxidations (Cavas and Yurdakoc 2005; Chew et al. 2008). Therefore, effective free radical scavenging molecules are needed by food and pharmaceutical industries. The extract of NOTL33 isolate exhibited effective free radical scavenging activity comparable with that of BHA. The free radical scavenging activity of the endophytic extracts could be attributed to the presence of phenolic compounds (Huang et al. 2007).

The in vivo plant pathogenicity of *B. ochroleuca* NOTL 33 strain had not been studied. Nevertheless, despite only few reports on the plant pathogenicity of *B. ochroleuca*, several reports describe the species as a bio-control agent against certain plant pathogens owing to its mycoparasitic nature (Burgess et al. 1997). Strain NOTL 33 was isolated as an endophyte from the disease symptomless host leaf, which shows the non-virulence nature of the strain. RNA secondary structure analysis revealed a clear distinction of NOTL 33 strain from the pathogenic strain (Fig. 4). Therefore, considering the in vitro antimicrobial and volatile antifungal activities of the strain, we may infer that strain *B. ochroleuca* NOTL 33 could be used as a bio-control agent against plant pathogen as well as post harvest pathogens.

The endophytic *B. ochroleuca* NOTL33 isolated from a medicinal tree growing in a biodiversity hotspot could be expected to be a distinct biotope. This study clearly demonstrates the capacity of the isolate to produce volatile antimycotic compounds and to produce secondary metabolites with significant antibacterial, antifungal and free radical scavenging activities. The results suggest that this isolate could be a potential source of antimicrobial compounds and a candidate to be developed as an effective biofumigant. However, further investigation is being carried out on the purification and characterisation of the active metabolites from this endophytic fungal isolate, which may lead to the discovery of novel bioactive molecules of industrial and pharmaceutical importance.

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