Mycoscience (2012) 53:113–121
DOI 10.1007/s10267-011-0146-z

FULL PAPER

Diversity and antimicrobial activity of endophytic fungi isolated from *Nyctanthes arbor-tristis*, a well-known medicinal plant of India

Surendra K. Gond · Ashish Mishra · Vijay K. Sharma · Satish K. Verma · Jitendra Kumar · Ravindra N. Kharwar · Anuj Kumar

Received: 18 December 2010 / Accepted: 19 July 2011 / Published online: 10 August 2011
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Abstract Endophytic fungi from *Nyctanthes arbor-tristis* were isolated and evaluated for their antimicrobial activity. A total of 19 endophytic fungi were isolated from 400 segments of healthy leaf and stem tissues of *N. arbor-tristis*. Eighteen endophytic fungi were obtained from leaf, while only ten from stem. *Alternaria alternata* had the highest colonization frequency (15.0%) in leaf, whereas *Cladosporium cladosporioides* ranked first in stem with a colonization frequency of 12%. The diversity and species richness were found higher in leaf tissues than in stem. The similarity indices between leaf and stem were 0.473 for Jaccard's and 0.642 for the Sorenson index, respectively. Of 16, 12 (75%) endophytic fungal extracts showed antibacterial activity against either one or more pathogenic bacteria. The endophytic *Nigrospora oryzae* showed maximum inhibition against *Shigella* sp. and *Pseudomonas aeruginosa*. The leaf endophytes *Colletotrichum dematium* and *Chaetomium globosum* exhibited a broad range of anibacterial activity and were active against *Shigella flexnii*, *Shigella boydii*, *Salmonella enteritidis*, *Salmonella paratyphi*, and *P. aeruginosa*. Nine out of 16 (56.25%) endophytic fungi exhibited antifungal activity to one or more fungal pathogens. *Colletotrichum dematium* inhibited 55.87% of the radial growth of the phytopathogen *Curvularia lunata*. The antimicrobial activity of these endophytic microorganisms could be exploited in the biotechnological, medicinal, and agricultural industries.

Keywords Biocontrol agent · *Colletotrichum dematium* · Colonization frequency · Fungal endophytes · Metabolites

S. K. Gond · A. Mishra · V. K. Sharma · S. K. Verma · J. Kumar · R. N. Kharwar (✉)
Mycopathology and Microbial Technology Laboratory, Department of Botany, Banaras Hindu University, Varanasi 221005, India
e-mail: rnkharwar@yahoo.com

A. Kumar
Department of Botany, Buddha P.G. College, Kushinagar, UP, India

Introduction

Endophytes are the microbes that reside inside healthy plant tissues without causing any overt negative impact on the host (Petrini 1991). This is a topographical term and includes bacteria, fungi, actinomycetes, and algae, which spend their whole life or a period of life cycle in the symplast or apoplast region of healthy plant tissues without producing any disease or clinical symptoms. On the basis of their nature, endophytes may be categorized in three groups: (1) pathogens of another host that are non-pathogenic in their endophytic relationship; (2) non-pathogenic microbes; (3) pathogens that have been rendered nonpathogenic but are still capable of colonization by selection methods or genetic alteration (Backman and Sikora 2008). Endophytes play a major role in plant community health by providing resistance to hosts against different biotic and abiotic stresses (Kharwar et al. 2008; Gond et al. 2010). Endophytes are viewed as an outstanding source of novel bioactive natural products because many of them occupy literally millions of unique biological niches (higher plants) growing in a variety of unusual environments (Verma et al. 2009). Over 8,600 bioactive metabolites of fungal origin have been described (Berdy 2005). In some cases, plant-associated fungi are able to make the same bioactive compounds as the host plant itself. One of the best examples of this is the discovery of gibberellins in *Fusarium fujikuroi* Nirenberg in the early 1930s. Eventually it was determined that the gibberellins are

one of only five classes of phytohormones that are to be found in virtually all plants. This observation led to the prospect that endophytic fungi, associated with *Taxus brevifolia*, may also produce taxol (Stierle et al. 1993). Taxol itself is the world's first billion dollar anticancer drug, and its main source is *Taxus* spp. Potentially, a fungal source of taxol would reduce its price and save the plant from extinction in some areas. The success of finding fungal taxol has produced a paradigm for still other bioactive compounds to be found in endophytic microbes.

Based on knowledge of the chemistry and biology of endophytes, the isolation of natural products can give us a platform to replace the existing synthetic drugs that provide resistance to pathogens and contaminate safe environments. The plant targeted for the isolation of endophytes in this study was *Nyctanthes arbor-tristis* L.

N. arbor-tristis (Oleaceae) is a well-known medicinal plant of India and Southeast Asia. This plant is commonly known as Harsinghar, Parijata, or Night Jasmine. It is also called 'tree of sorrow' because Nyctanthes means 'night flowering' and arbor-tristis means 'the sad tree' as the flower loses its brightness during the daytime (Sasmal et al. 2007). *N. arbor-tristis* is a shrub or small tree up to 10 m height with gray or greenish rough bark with stiff, whitish hairs; young branches are sharply quadrangular. In addition, this plant is a native to India and grows luxuriantly in all parts of the country. The plant is widely used in India for its medicinal values. The flowers and leaves of *N. arbor-tristis* are well known for their interesting antibacterial, antifungal, antileishmanial, and cytotoxic activity. The local people of Chhattisgarh in India use seeds of this plant for the successful treatment of piles. The leaves and flowers are used in the treatment of gout. A significant antibacterial and cytotoxic activity was observed by the flower extract of this plant from Bangladesh (Khatune et al. 2001). Keeping in view the medicinal values of this host, the objectives of the work reported in this article were to isolate the endophytic fungi from leaf and stem tissues of the *N. arbor-tristis*, and to evaluate their potential as biocontrol agents against a range of pathogenic bacteria and fungi. This work is the first report of the incidence of endophytic fungi from *N. arbor-tristis* and their activity against other microbes that are pathogenic to both humans and plants.

Materials and methods

Plant selection site

Nyctanthes arbor-tristis, growing in the botanical garden of Banaras Hindu University (BHU), Varanasi (25.5°N 82.9°E, elevation 279 ft/85 m), India, was identified on the basis of external morphological characters. This plant was grown from seed in BHU nursery 12 years ago and was transplanted in the botanical garden to study its medicinal properties. Collection of leaf and stem was made randomly from the branches at the height of 3 and 1.5 m above the ground, respectively. All samples were collected in sterile polythene bags and brought to the laboratory in an icebox. Samples were stored at 4°C and processed for isolation within 48 h from collection. A total of 400 tissue segments (200 each from leaf and stem) were plotted for isolation of endophytic fungi.

Surface treatment, isolation, and identification

Samples were washed thoroughly in running tap water for 10 min to remove debris and finally washed with double distilled water to minimize the microbial load from the sample surface. The surface treatment was done adopting the methodology of Petrini et al. (1992), and the effectiveness of surface sterilization was checked according to the method of Schulz et al. (1993). Epiphytic mycelia were removed by immersing the tissues in 70% ethanol for 1–3 min and in aqueous solution of sodium hypochlorite (4% available chlorine) for 3–5 min followed by washing with 70% ethanol for 5–10 s. The tissues were then rinsed in sterile distilled water and allowed to surface dry in sterile conditions. The outer bark was removed, and the inner bark containing cortex was carefully dissected into small pieces (0.5×0.5 cm^2). The surface-sterilized leaf was also cut into small pieces of 0.5×0.5 cm^2. The pieces were placed in petri dishes containing potato dextrose agar (PDA) medium supplemented with streptomycin (250 mg/l) and incubated for 20 days at 25 ± 2°C in a BOD cum humidity incubator (Calton Super Delux, NSW, New Delhi). Tissues were observed for fungal growth at alternate day intervals for 20 days. Actively growing fungal tips emerging from plant tissues were sub-cultured on PDA petri plates for identification and enumeration. The endophytic fungi were identified according to their macro- and microscopic structures. Species-level identification was done by using standard manuals described by Barnett and Hunter (1998), Ellis (1976), Von Arx (1978), Raper and Thom (1949), and Ainsworth et al. (1973). All isolated and identified endophytic fungi were assigned specific code numbers (MMTL: NAT 001-NAT 407) and maintained in cryovials on PDA layered with glycerol (15%, v/v), and also in lyophilized form at −20°C in a deep freezer (Blue Star). All the samples were deposited at the Department of Botany, Banaras Hindu University, India.

Antibacterial activity of endophytic fungi

The isolated endophytic fungi were evaluated for their antibacterial activity against eight species of human

pathogenic bacteria. All human pathogenic bacteria were obtained from the Institute of Medical Sciences (IMS), BHU, Varanasi. The isolated endophytic fungi were grown in 2 l Erlenmeyer flasks containing 1 l of potato dextrose broth (PDB) and incubated at 25 ± 2°C for 21 days. The fermented broths were extracted twice with the same volume of ethyl acetate. The extracts were combined and evaporated to dryness in vacuo by a rotary evaporator (Rotary vacuuma, Perfit India, Ltd). The inhibitory effect of the extract obtained from endophytic fungi was tested by a modified Bauer-Kirby method (Bauer et al. 1966) with paper discs. The collected crude extract was weighed and finally dissolved in methanol and diluted to 0.5 mg/µl for assay. A sterile paper disc (5 mm diameter, Whatman no. 1) was impregnated with 10 µl of methanolic extract using a micropipette and kept under a laminar hood for 20 min to dryness. The air-dried paper discs containing 5 mg crude extract were used to test the activity against clinical isolates of eight human pathogenic bacteria (*S. flexnii* IMS/GN1, *S. boydii* IMS/GN2, *S. enteritidis* IMS/GN3, *S. paratyphi* IMS/GN4, *P. aeruginosa* ATCC 27853, *Citrobacter freundii* IMS/GN5, *Morganella morganii* IMS/GN6, and *Proteus vulgaris* IMS/GN7). The bacterial culture was streaked evenly with a cotton swab onto the surface of solidified Mueller-Hinton (MH) agar petri plates. The paper discs containing 5 mg crude extract were placed on the surface of the Mueller-Hinton medium seeded with test bacterium in separate petri plates. The paper disc dried after impregnating with only methanol of the same volume was considered as control. The reference antibiotic discs were ampicillin (10 µg/disc) and ciprofloxacin (5 µg/disc) from HiMedia Laboratories Pvt. Ltd., India. The plates were incubated at 35 ± 2°C for 24 h and measured for inhibition zones. Each test was done in three replicates.

Antifungal activity of endophytic fungi

Antagonistic activity of the isolated endophytic fungi was observed against eight pathogenic (6 phytopathogens and 2 human pathogens) fungi. The phytopathogens were *A. alternata* (Fr.) Keissl. IAS/RC-1, *Bipolaris* sp. IAS/RC-2, *C. cladosporioides* (Fresen.) G.A. de Vries MMTL/PP-1, *Curvularia lunata* (Wakker) Boedijn MMTL/PP-2, *Fusarium oxysporum* Schltdl. IAS/RC-3, and *Fusarium udum* E.J. Butler MMTL/PP-3, and human pathogens were *Microsporum gypseum* E. Bodin IMS/A-014 and *Trichophyton rubrum* (Castel.) Sabour. IMS/2013. We took six fungal phytopathogens from Institute of Agricultural Sciences, BHU, and two fungal human pathogens from IMS, BHU, Varanasi.

The inhibition of fungal pathogens by the test antagonist's endophyte was examined on PDA plates using the dual culture technique. Five-millimeter-diameter mycelial plugs of actively growing endophytic fungi were placed at the periphery of the culture plate and incubated for 2 days at 25 ± 2°C. After 2 days, the plate was doubly inoculated with another 5-mm-diameter mycelial plug of the pathogen placed 5 cm from the test antagonist. The dual culture plates were incubated for an additional 7 days at 25 ± 2°C. The percentage inhibition of the growth of the pathogen was calculated with the help of the formula given by Whipps (1997).

$$\% \text{ inhibition of radial growth} = \frac{R_1 - R_2}{R_1} \times 100$$

where R_1 is the farthest radial distance grown by the pathogen in the opposite direction of the antagonist, and R_2 is the distance grown on a line between the inoculation of the pathogen and the antagonist. Each test was done in three replicates.

Statistical analysis

The colonization 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 of plant tissue colonized by each fungus and N_t = total number of segments of plant tissue studied.

The similarity of endophytic fungal assemblages among both tissues was compared using the following similarity indices:

Sorensen's index of similarity (QS), $QS = 2a/(2a + b + c)$, where '*a*' is the number of common species in both endophytic populations, while '*b*' and '*c*' are the number of species specified to leaf and stem, respectively (Osono and Mori 2004). Jaccard's index of similarity (JS) was calculated using the formula: $JS = a/a + b + c$, where '*a*' is the number of common species in both myco-populations, while '*b*' and '*c*' are the number of species specified to leaf and stem, respectively.

The fungal diversity of both endophytic myco-populations was estimated with the following diversity indices. The reason for using these diversity indices was to take advantage of the strengths of each index and to predict the complete structure of both myco-populations. Simpson's index of dominance (D) was calculated by the following formula (Simpson 1951).

$D = \sum(n/N)^2$, where n = the total number of isolates of a particular species, while N = the total number of isolates of all species.

Simpson's diversity index = 1D. Species richness = $S/\sqrt{N}$, where S = total number of species. The Shannon-Wiener index (H') was calculated by $\sum$(pi ln pi), where pi = n/N. Species evenness E was also evaluated by $H'/\ln S$.

The range of antibacterial activity of the endophytic fungal metabolite was calculated as the number of bacteria inhibited by each fungal metabolite divided by the total number of bacteria tested and multiplied by 100.

Results

Endophytic fungi

Endophytic fungi were isolated from healthy, symptomless leaf and stem segments of *N. arbor-tristis* followed by the proper surface sterilization. A total of 19 endophytic fungal species were isolated from 400 segments of both leaf and stem tissues of *N. arbor-tristis* (Table 1). Eighteen endophytic fungal species under 15 taxa and 10 species under 9 taxa, respectively, were isolated from 200 each of leaf and stem segments. In terms of total isolates under all endophytic taxa, 281 isolates were recovered from leaf and 126 from stem tissues. In leaf tissues *A. alternata* showed highest colonization (15.0%) frequency, while in stem, *C. cladosporioides* ranked first for colonization (12%) frequency (Table 1). *Curvularia fallax* Boedijn and *F. oxysporum* showed the least colonization frequency (1.5%) in leaf. In stem segments, *F. oxysporum* and *Phomopsis* sp. showed the lowest colonization frequency (2.5%) (Table 1). The common species found in both tissue segments were *A. alternata*, *C. cladosporioides*, *C. lunata*, *Curvularia oryzae* Bugnic., *F. oxysporum*, *Acremonium* sp., *N. oryzae* (Berk. & Broome) Petch, *Phomopsis* sp., and *Rhizoctonia* sp. (Fig. 1). Two species of cosmopolitan aspergilla (*Aspergillus fumigatus* Fresen. and *Aspergillus niger* Tiegh.) were isolated only from leaf segments, while *Drechslera ellisii* Danquah was restricted only to the stem tissues. Only a single member of the ascomycete *C. globosum* Kunze was found to be colonized in the leaf. Two unidentified species (NAB1 and NAB2), confined only to leaf segments, were isolated. NAB1 showed a compact and white colony, whereas NAB2 showed a discrete and brown colony on PDA. These members may be from the group of Mycelia-Sterilia, which did not produce any asexual or sexual propagules either on synthetic or host extract supplemented growth media. The result shows that colonization of endophytic fungi was greater and more diverse in leaf than in stem tissues (Table 1).

The diversity of the endophytic community isolated from both tissues was compared using indices of α-diversity (Shannon-Wiener index and Simpson's diversity index) and their components, i.e., species richness and evenness (Table 2). The concentration of dominance or Simpson's dominance of endophytic fungi was higher in

Table 1 Endophytic fungi isolated from leaf and stem tissues of *Nyctanthes arbor-tristis*

Endophytic fungi	Leaf		Stem		Total
	I	%CF	*I*	%CF	%CF
A. alternata	30	15.0	12	6.0	10.50
Aspergillus fumigatus	20	10.0	0	0.0	5.00
A. niger	25	12.5	0	0.0	6.25
C. cladosporioides	26	13.0	24	12.0	12.50
C. dematium	21	10.5	0	0.0	5.25
C. globosum	13	6.5	0	0.0	3.25
C. lunata	18	9.0	22	11.0	10.00
C. oryzae	9	4.5	8	4.0	4.25
C. fallax	3	1.5	0	0.0	0.75
D. ellisii	0	0.0	9	4.5	2.25
F. oxysporum	3	1.5	5	2.5	2.00
Humicola grisea	19	9.5	0	0.0	4.75
Acremonium sp.	26	13.0	14	7.0	10.00
N. oryzae	22	11.0	17	8.5	9.75
Penicillium sp.	9	4.5	0	0.0	2.25
Phomopsis sp.	14	7.0	5	2.5	4.75
Rhizoctonia sp.	12	6.0	10	5.0	5.50
NAB1	7	3.5	0	0.0	1.75
NAB2	4	2.0	0	0.0	1.00

I no. of isolates, *CF* colonization frequency

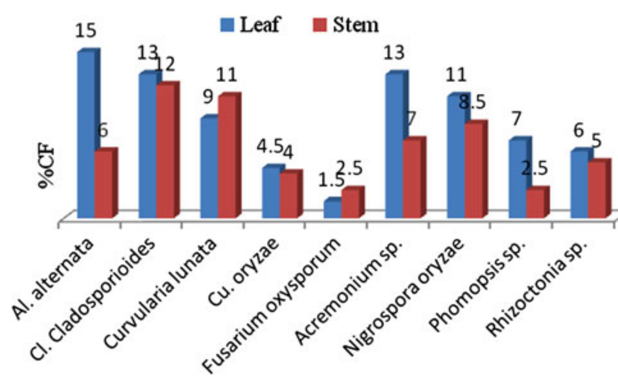

Fig. 1 Colonization frequency of endophytic fungi common to leaf and stem tissues of *Nyctanthes arbor-tristis*

Table 2 Diversity indices of endophytic fungi colonizing leaf and stem tissues of *Nyctanthes arbor-tristis*

Indices	Leaf	Stem
Simpson's dominance	0.0714	0.1249
Simpson's diversity	0.9286	0.8750
Species richness	1.0738	0.5966
Shannon-Wiener	2.7303	2.1796
Evenness (*E*)	0.9446	0.9466

For calculation of these indices, see "Statistical analysis" in "Materials and methods"

Table 3 Jaccard's (Jc) and Sorenson's similarity indices for endophytic fungi of leaf and stem of *N. arbor-tristis*

	Jaccard's index Leaf	Sorenson's index Stem
Leaf	1.000	0.642
Stem	0.473	1.000

For calculation of these indices, see "Statistical analysis" in "Materials and methods"

stem tissues. Both Simpson's and Shannon-Wiener diversity indices were higher in fungal endophytes of leaf tissues. The species richness was also greater in endophytic fungi colonizing leaf tissues. There was little difference between the species evenness to endophytes of both tissues (Table 2). Jaccard's (Jc) and Sorenson's similarity indices between the endophytes of both tissues were 0.473 and 0.642, respectively (Table 3).

Antibacterial activity of endophytic fungi

Sixteen endophytic fungi isolated from *N. arbor-tristis* were tested for antibacterial activity by disc diffusion assay against eight clinical isolates of human pathogenic bacteria (*S. flexnii*, *S. boydii*, *S. enteritidis*, *S. paratyphi*, *P. aeruginosa*, *C. freundii*, *M. morganii*, and *P. vulgaris*). Of 16, 12 (75%) endophytic fungi showed antibacterial activity against one or more than one bacteria (Table 4). *Colletotrichum dematium* (Pers.) Grove and *C. globosum* exhibited a high range (62.5%) of antibacterial activity against the tested pathogens (Fig. 2). These two species were active against *S. flexnii, S. boydii*, *S. enteritidis*, *S. paratyphi*, and *P. aeruginosa*. *Nigrospora oryzae* gave the maximum inhibition zone (22 mm) against *S. paratyphi* (Table 4). *Shigella flexnii*, *S. boydii*, and *P. aeruginosa* were also maximally inhibited by *N. oryzae*. *Citrobacter freundii*, *M. morganii*, and *P. vulgaris* were resistant against all endophytic fungal extracts. *S. boydii* was found to be most susceptible and was inhibited by ten endophytic fungal extracts.

Antifungal activity of endophytic fungi

Antagonistic activity of 16 endophytic fungi was also evaluated against 8 pathogenic fungi. Nine out of 16 endophytic fungi exhibited antifungal activity against one or more fungal pathogens. The most prominent activity was shown by *C. dematium* against *C. lunata*, giving 55.87% radial growth inhibition in dual culture (Table 5). *Colletotrichum dematium* also inhibited the growth of maximum numbers (5 out of 8) of fungal pathogens. *Acremonium* sp. showed 53.17% radial growth inhibition to *C. cladosporioides*. *Acremonium* sp. and *N. oryzae* inhibited the growth of three out of eight pathogenic fungi. The most susceptible phytopathogen was *C. cladosporioides,* whose growth was inhibited up to 53.17% by *Acremonium* sp., 39.70% by *A. fumigatus*, 39.66% by *F. oxysporum*, 39.57% by *C. dematium*, and 31.60% by *D. ellisii*, respectively. The growth of the human pathogen *M. gypseum* was inhibited 38.8% by *N. oryzae* and 35.13% by *C. dematium*. The other human pathogen, *T. rubrum*, was inhibited by *C. globosum* and *Acremonium* sp. The endophytic *Rhizoctonia* sp. showed antifungal activity only against *C. lunata* giving 45.44% radial growth inhibition (Table 5).

Discussion

Endophytes colonize inside healthy plant tissues to get nutrition and shelter from the host, and in response produce many functional metabolites, which may enhance the host fitness, and have anti-feedant activity and provide resistance against various biotic and abiotic stresses as well. Fungal endophytes are relatively less explored and a new addition to the available diversity of fungi (Kharwar et al. 2009b). Earlier it was thought that one plant can be a habitat of six fungi, but after including fungal endophytes, the ratio of fungal:plant species has now been changed to 33:1 (Hawksworth and Rossman 1997). Conducting a comprehensive study of any host plant is necessary before screening the potential of endophytic fungi. The most frequently isolated genera in this study were *A. alternata* and *C. cladosporioides* (Table 1). These genera are common epiphytes, but can also occur as endophytes; this result supports many earlier works (Bacon and White 2000; Kharwar et al. 2010), and surprisingly, these two genera also dominated over the most cosmopolitan species of aspergilli (Table 1), which suggests that it may be due to substrate specificity. All isolated fungi except *C. globosum* were members of the fungal subdivision Deuteromycotina. Anamorphic fungi occur as a major assemblage in the counting of endophytic fungi (Gond et al. 2007; Verma et al. 2007). Fungal endophytes belonging to Deuteromycotina in mangrove vegetations of costal Karnataka, Picchavaran, and Pondicherry (India) were also more prevalent than the Ascomycotina (Suryanarayanan et al. 1998; Maria and Sridhar 2003). Hyphomycetes, a class of Deuteromycotina, ranked first, followed by Coelomycetes, Blastomycetes, and Mycelia-Sterilia. Hyphomycetous fungi are common endophytes among plants inhabiting temperate, tropical, and rainforest vegetations (Bacon and White 1994). Among stem endophytes, except for *D. ellisii*, all others were commonly isolated from both tissues, and this may be a fine example of tissue specificity. Leaf harbored a greater number of endophytic fungi with high diversity than stem (Tables 1, 2), and this may be due to the large

Table 4 Antibacterial activity of endophytic fungi (crude extract 5 mg per disc) of *N. arbor-tristis*

	Isolate no.	Diameter of inhibition zone (mm ± SD) of human pathogenic bacteria*							
		A	B	C	D	E	F	G	H
Endophytic fungi									
A. alternata	MMTL: NAT 22	6.33 ± 0.57	11.33 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	7.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. cladosporioides	MMTL: NAT130	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
C. dematium	MMTL: NAT152	13.66 ± 0.57	14.00 ± 0.00	7.33 ± 0.57	8.66 ± 0.57	11.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. globosum	MMTL: NAT160	13.00 ± 0.00	14.00 ± 1.00	6.00 ± 0.00	6.00 ± 0.00	13.66 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. lunata	MMTL: NAT210	0.00 ± 0.00	7.66 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. oryzae	MMTL: NAT215	0.00 ± 0.00	6.33 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. fallax	MMTL: NAT230	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
D. ellisii	MMTL: NAT238	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
F. oxysporum	MMTL: NAT242	6.33 ± 0.57	7.00 ± 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
H. grisea	MMTL: NAT265	0.00 ± 0.00	10.66 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Acremonium sp.	MMTL: NAT290	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	13.33 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
N. oryzae	MMTL: NAT341	15.00 ± 0.00	18.00 ± 1.00	0.00 ± 0.00	22.00 ± 1.00	15.66 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Phomopsis sp.	MMTL: NAT377	0.00 ± 0.00	8.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	10.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Rhizoctonia sp.	MMTL: NAT394	6.66 ± 0.57	12.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.33 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
NAB1	MMTL: NAT402	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	12.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
NAB2	MMTL: NAT407	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
Controls									
Methanol		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
Ampicillin (10 μg/disc)		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
Ciprofloxacin (5 μg/disc)		18.00 ± 0.00	39.00 ± 0.00	40.00 ± 0.00	40.00 ± 0.00	34.00 ± 0.00	32.00 ± 0.00	30.00 ± 0.00	32.00 ± 0.00

A *S. flexnii* IMS/GN1, B *S. boydii* IMS/GN2, C *S. enteritidis* IMS/GN3, D *S. paratyphi* IMS/GN4, E *P. aeruginosa* ATCC 27853, F *C. freundii* IMS/GN5, G *M. morganii* IMS/GN6, H *P. vulgaris* IMS/GN7, mm ± SD, millimeter ± standard deviation

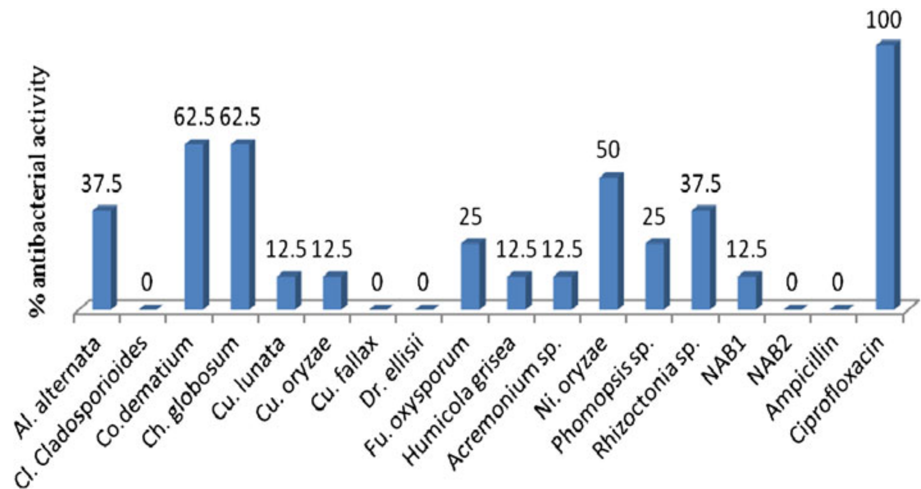

Fig. 2 Bacterial inhibition potential of endophytic fungi of *Nyctanthes arbor-tristis.* Calculated as number of bacteria inhibited by each fungal metabolite divided by total number of bacteria tested and multiplied by 100

Table 5 Antifungal activity of endophytic fungi of *N. arbor-tristis*

Endophytic fungi	Isolate no.	% growth inhibition of pathogenic fungi							
		A	B	C	D	E	F	G	H
A. alternata	MMTL: NAT22	–	–	–	–	–	–	–	–
A. fumigatus	MMTL: NAT44	–	39.70	–	32.02	–	–	44.45	38.94
C. globosum	MMTL: NAT160	–	–	–	–	–	–	44.45	38.94
C. cladosporioides	MMTL: NAT130	–	–	–	–	–	–	–	–
C. dematium	MMTL: NAT152	23.57	39.57	–	37.23	35.15	–	55.87	–
C. lunata	MMTL: NAT210	–	–	–	–	–	–	–	–
C. oryzae	MMTL: NAT215	–	–	–	–	–	–	–	–
C. fallax	MMTL: NAT230	–	–	–	–	–	–	–	–
D. ellisii	MMTL: NAT238	–	31.60	–	–	–	–	33.53	-
F. oxysporum	MMTL: NAT242	33.37	39.66	–	–	–	–	-	-
H. grisea	MMTL: NAT265	–	–	–	–	–	–	–	–
Acremonium sp.	MMTL: NAT290	40.33	53.17	–	–	–	–	–	34.45
N. oryzae	MMTL: NAT341	38.94	–	–	–	38.80	36.83	–	–
Rhizoctonia sp.	MMTL: NAT394	–	–	–	–	–	–	45.44	–
NAB1	MMTL: NAT402	–	–	–	–	–	–	–	–
NAB2	MMTL: NAT407	–	–	38.47	–	–	40.88	–	–

A *A. alternata* IAS/RC-1, B *C. cladosporioides* MMTL/PP-1, C *F. oxysporum* IAS/RC-3, D *Fusarium udum* MMTL/PP-3, E *M. gypseum* IMS/A-014, F *Bipolaris* sp. IAS/RC-2, G *C. lunata* MMTL/PP-2 and H *T. rubrum* IMS/2013

surface area exposed to the outer environment and the presence of stomata providing passage to the entry of fungal mycelia. This may also be one of the reasons why most endophytes of leaf had greater colonization frequency than that of stem (Fig. 1). In contrast to the higher diversity in leaf, Kumar and Hyde (2004) have reported the highest Shannon diversity index for endophytic fungi in twig xylem followed by that of leaf of *Tripterygium wilfordii*. The high species richness in leaf corroborates the findings of Verma et al. (2007) for *Azadirachta indica*. Endophytes are regarded as latent pathogens, which may be pathogenic after receiving a favorable outer environment or in plant disease conditions. The high diversity of endophytes in leaf may also act as saprophytes in certain cases to enhance the litter degradation when leaf senescence occurs or plants die (Promputtha et al. 2007).

Currently, there is demand for a search for new antimicrobial agents because of the development of pathogen resistance to available drugs. People are generating new synthetic drugs, but these may have an adverse effect on the environment. The antibacterial activity of endophytic metabolites was screened against clinical isolates of human pathogenic bacteria. All tested endophytic fungal metabolites except *C. cladosporioides*, *D. ellisii*, and NAB2 showed the significant antibacterial activity either to one or more pathogenic bacteria (Table 4). About 75% of

endophytic fungi showed antibacterial activity, which supports the view of Schulz et al. (2002), while it differs from another study where only 8.3% of isolates of endophytic fungi from *Dracaena cambodiana* and *Aquilaria sinensis* showed antimicrobial activity (Gong and Guo 2009). *Colletotrichum dematium*, *C. globosum*, and *N. oryzae* emerged as more efficacious isolates that exhibited a broad range of antibacterial and antifungal activity as well (Fig. 2; Table 5), and interestingly, some earlier researchers have also isolated many antimicrobial compounds, such as colletotric acid from *Colletotrichum* sp. and fusaruside from *Fusarium* sp. (Zou et al. 2000; Shu et al. 2004). Surprisingly, over 100 anticancer compounds have been reported within a couple of decades from fungal endophytes, and a few of them were recovered in our study, which indicates that if they are evaluated critically, they may act as a source of either antibacterial or anticancer compounds (Kharwar et al. 2011). An endophytic strain of *Chaetomium* sp. isolated from *Nerium oleander* showed a strong antioxidant potential with an IC_{50} value of 109.8 μg/ml (Huang et al. 2007).

Since *N. oryzae* produced the maximum inhibition (22 mm) against *S. paratyphi*, a causal agent of typhoid fever in humans, this is an indication for the isolation of a strong antibacterial compound for this pathogen. *N. oryzae* produced a 15-mm inhibition zone against *S. flexnii*, while the synthetic antibiotic ciprofloxacin (5 μg/disc) produced an 18-mm inhibition zone, showing a very encouraging and positive result, and the purified active principle of this metabolite can provide a good alternative natural source for an antibiotic like ciprofloxacin, etc. Our result corroborates the earlier study where partially purified extracts from solid-state fermentation of endophytic *N. oryzae*, *Alternaria* sp. and *Papulospora* sp. isolated from medicinal plants of the Western Ghats of India also showed an inhibitory activity against both gram-positive and -negative bacteria (Raviraja et al. 2006). Since no bacterial pathogen was inhibited by ampicillin (10 μg/disc), this may be due to the development of resistance against this antibiotic as ampicillin-resistant *Shigella* sp. produces *β*-lactamase (Smith et al. 1974). The continuous exposure of bacteria to the available antimicrobial drug also enhances the opportunity to develop resistance against a particular antimicrobial agent. As per our findings, *C. cladosporioides* had maximum colonization frequency, but failed to produce even mild antibacterial activity, and this observation may suggest that there is no direct relationship between higher fungal colonization frequency and antibacterial activity in this plant. Although *C. cladosporioides* did not show any antibacterial activity in this experiment, while in a recent study, Zhang et al. (2011) reported the production of an alkaloid, huperzine A, used in treating the Alzheimer's disease. A potential antibacterial naphthaquinone, 'javanicin,' was purified from an endophytic fungus *Chloridium* sp. of *A. indica* by our laboratory (Kharwar et al. 2009a). A new cerebrosides named fusaruside was characterized from the chloroform-methanol (1:1) extract of *Fusarium* sp., IFB-121, an endophytic fungus of *Quercus variabilis* with strong antibacterial and xanthine oxidase inhibitory activity (Shu et al. 2004). The prominent antifungal activity of endophytic *C. dematium* shows the potential of extracting antifungal compounds (Table 5). In an earlier study in our laboratory, 50% of the tested endophytic fungi from *Eucalyptus citriodora* showed antagonistic activity against a variety of fungi representing pathogens to both humans and plants (Kharwar et al. 2010). Besides the antimicrobial and other bioactivities, endophytic fungi are also reported to produce biodiesel (Strobel et al. 2008), which may be the future hot cake for endophytic study. In fact, this is probably the most comprehensive collection of endophytic fungi from *N. arbor-tristis* in the world, and perhaps this study is also the first of its kind with this host. Interestingly, the findings of this study provide a strong platform for the isolation and purification of novel natural antimicrobial agents from endophytic fungi of *N. arbor-tristis*.

Acknowledgments The authors thank the head of the Department of Botany (Prof. B.R. Chaudhary), BHU Varanasi, for providing the necessary facilities. SKG acknowledges the help of CSIR, New Delhi, for upgrading JRF to SRF. We extend our thanks to Prof. Gopal Nath and Dr. Ragini Tilak (IMS, BHU) for providing human bacterial and fungal pathogens and also to Prof. Ramesh Chand (Inst. Ag. Sc., BHU), for phytopathogens. RNK extend his thanks to DST, New Delhi, for financial help (file no. SR/SO/PS-2009, dt-10-5-2010).

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