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ORIGINAL ARTICLE

Diversity and antimitotic activity of taxol-producing endophytic fungi isolated from Himalayan yew

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Abstract Endophytic fungi represent an under explored resource of novel lead compounds and have the capacity to produce diverse classes of plant secondary metabolites. Here, we investigated the endophytic fungal diversity of taxol-producing endophytes from *Taxus baccata* L. ssp. *wallichiana* (Zucc.) Pilger and also tested the antimitogenic effect of fungal taxol using potato disc tumor assay. A total of 60 fungal endophytes were isolated from the inner bark (phloem-cambium) of *T. baccata* ssp. *wallichiana*, collected from different locations of the northern Himalayan region. Two key genes, *DBAT* (10-deacetylbaccatin III-10-*O*-acetyl transferase) and *BAPT* (C-13 phenylpropanoid side chain-CoA acyltransferase), involved in taxol biosynthesis were used as molecular markers for the screening of taxol-producing strains. Five representative species gave positive amplification hits by molecular marker screening with the *bapt* gene. These fungi were characterized and identified based on morphological and molecular identification. The taxol-producing capability of these endophytic fungi was validated by HPLC-MS. Among the five taxol-producing fungi, the highest yield of taxol was found to be 66.25 μg/l by *Fusarium redolens* compared with those of the other four strains.

Keywords *Taxus baccata* · Taxol · Endophytic fungi · *Fusarium* spp. · *Microdiplodia* sp. · *Paraconiothyrium brasiliense* · *Agrobacterium tumefaciens*

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Introduction

Among plant-derived natural products, taxol (a highly efficient, low toxicity and broad-spectrum natural anti-cancer drug) is widely and successfully used for clinical application against different types of cancer (McGuire et al. 1989; Rowinsky et al. 1990; Holmes et al. 1991; Yunan et al. 2000; Pandey et al. 2003). This tubulin-binding diterpenoid alkaloid was originally obtained from the bark of the Pacific yew tree *Taxus brevifolia* by Wani et al. (1971). The main natural source of taxol is the bark of yew trees (*Taxus* species) wherein it exists in low concentrations, i.e. 13,500 kg of *T. brevifolia* bark yields only 1 kg of taxol (Wheeler et al. 1992; Pezzuto 1996; Nadeem et al. 2002). Furthermore, trees belonging to *Taxus* species are rare and very slow growing, and the traditional methods of extracting taxol from the bark are inefficient and environmentally costly, causing irreplenishable damage and loss of the endangered natural source, and even the yields of pure drug are low. With the increasing demand for taxol and a shortage of plant resource, there is an urgent need to find alternative production methods. Several alternative strategies have been developed for the production of taxol, including tissue culture of *Taxus* species and chemical synthesis. However, these different production alternatives are not able to meet the increasing taxol demand and are very complex, tedious, and uneconomical (Nicolaou et al. 1994; Collin 2001). Consequently, more production options are still required to lower the price of taxol and to increase its availability.

Taxomyces andreanae obtained from the Pacific yew is the first report of a microbial taxol producer (Stierle et al. 1993). This finding implies that entophytic fungi serve as a potential and the most desirable means of taxol production. The advantages of microbial taxol production include fast growth, easy genetic manipulation, and the possibility of process optimization on an industrial level (Flores-Bustamante et al. 2010). Isolation of endophytic fungi from plant material is a comparatively simple

process, but screening is laborious and time consuming (Zhou et al. 2007). Compared to biochemical screening methods (traditional screening), molecular marker screening is a rapid and efficient alternative method for the detection of endophytes capable of taxol production. Primers based on two key genes of the taxol biosynthetic pathway, 10-deacetylbaccatin III-10-*O*-acetyl transferase (DBAT) and C-13 phenylpropanoid side chain-CoA acyltransferase (BAPT), have been applied in the primary screening of taxol-producing endophytic fungi (Zhang et al. 2008). In the past two decades, several endophytic micro-organisms isolated from different geographical settings have been reported to produce taxol through biochemical or molecular marker screening (Flores-Bustamante et al. 2010), the majority of which belongs to *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Monochaetia*, *Ozonium*, *Pestalotiopsis*, *Pithomyces*, *Taxomyces*, *Tubercularia*, etc (Stierle et al. 1993; Gangadevi and Muthumary 2008; Zhao et al. 2008, 2009; Zhou et al. 2010). Although the amount of taxol found in most of the *Taxus*-associated endophytic fungi is small compared to that of trees, the short generation time and high growth rate of the fungi make it worthwhile to investigate these species for taxol production (Liu et al. 2009).

In the present work, we investigated the endophytic taxol-producing endophytic fungal diversity of *Taxus baccata* L. ssp. *wallichiana* (Zucc.) Pilger (Himalayan yew). *Taxus baccata* ssp. *wallichiana* is the only species of *Taxus* which is found in the temperate Himalayas at altitudes of 1,800–3,300 m amsl. It is a medium-sized, slow-growing, non-resinous, evergreen conifer that undergoes cross-pollination and has been found to grow best in well-drained moist areas, in cool temperate to sub-tropical climates. To the best of our knowledge, no work to study the diversity of taxol-producing endophytic fungi from this yew species growing in the northern Himalayan region of India has been reported to date. The key genes involved in taxol biosynthesis were used as molecular markers for screening fungal isolates. The antimitotic activity of taxol produced by the fungi was tested by potato disc induction assay using *Agrobacterium tumefaciens* as the tumor-inducing agent.

Materials and methods

Isolation of endophytic fungi

Bark samples were collected from the stems of *T. baccata* ssp. *wallichiana* growing at different locations of the northern Indian Himalayan region. Samples were collected from Bhadrewah (district Doda, Jammu and Kashmir), Shimla (Himachal Pradesh), and Almora (Uttrakhand) during different months of the year. The bark samples were surface-sterilized by treating with 75 % aqueous ethyl alcohol (v/v) for 60 s to kill epiphytic micro-organisms, followed by washing in 4 % sodium hypochlorite for 60–90 s and rinsing twice in sterilized distilled water. The excess moisture on the bark surface was blotted using sterile filter paper. Surface-disinfected small pieces of inner bark were excised and placed on the surface of potato dextrose agar (PDA) medium supplemented with ampicillin (50 μg/ml) in Petri plates, incubated at 25–28 °C for 5–10 days to allow the growth of endophytic fungi. The plates were periodically checked for the growth of endophytic fungal colonies and culture purity. Pure fungal cultures of endophytic isolates were obtained by the hyphal tip method (Strobel et al. 1996). All the fungal isolates were coded and stored in sterile distilled water as agar plugs.

Primary screening of taxol-producing fungi based on PCR amplification

The fungal isolates were inoculated aseptically and individually in 20 ml of potato dextrose broth in 150-ml Erlenmeyer flasks. Cultures were incubated at 25–28 °C at 120 rpm for 3–5 days and the mycelium of each fungus was harvested by centrifugation at 12,000 rpm for 10 min. Genomic DNA was extracted from the mycelia using the CTAB method (Zhang et al. 1996). The conserved sequences of two key genes involved in the taxol biosynthetic pathway, *dbat* and *bapt*, were used as molecular markers for the primary screening of taxol-producing fungi. The following specific primers, *dbat*-F 5′-GGGAGGGTGCTCTGTTTG-3′, *dbat*-R 5′-GTTACCTG AACCACCAGAGG-3′, and *bapt*-F 5′-CCTCTCTCCGCC ATTGACAA-3′ and *bapt*-R 5′-TCGCCATCTCTGCCATAC TT-3′, as described by Li et al. (2006), were used for PCR amplification, which was performed in the GeneAmp® PCR system 2700 (Applied Biosystems, USA). The fungal isolates were first screened for the presence of the *dbat* gene and then screened for the *bapt* gene. PCR amplification was carried out as per the previously reported PCR conditions (Zhang et al. 2008). The amplified DNA fragments were analysed by agarose gel electrophoresis and the amplified products were purified using QIAquick® PCR purification kit (QIAGEN). Purified PCR products were ligated to pTZ 57R/T vector, transformed into *E. coli* DH5α. Transformed colonies were carefully picked and the inserts were sequenced. Those fungi which gave PCR positive results for both the molecular markers were first identified and then subjected to biochemical screening.

Identification of endophytic fungi

The selected fungal isolates were subcultured onto fresh PDA medium and incubated at 28 °C for 2 weeks. These isolates were identified based on the morphology of the fungal colony, the characteristics of the fungal spores, and molecular phylogenetic analysis. Using the previously isolated fungal genomic DNA, fungal internal transcribed spacer (ITS) fragments

were amplified using ITS1 (5′-TCCGTAGGTGAACCTG CGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (White et al. 1990) as described in Pandey et al. (2003). The ITS sequences were compared with National Center for Biotechnology Information database using BLAST search to find the possible homologous sequences of the newly sequenced taxa for each fungus. CLUSTAL W software was used to generate alignments of the endophytic fungi (Larkin et al. 2007). The phylogenetic analysis was carried out by the maximum parsimony method and the Kimura two-parameter distance calculation by MEGA5 software. The bootstrap was 1,000 replications to assess the reliable level for the nodes of the tree (Tamura et al. 2011). Cultures of selected fungal endophytes were deposited in the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. The accession numbers for TBPJ-B, B-7, TBPJ-A, TBPJ-13, and C-1 are MTCC11742, MTCC11754, MTCC11759, MTCC11758, and MTCC11754, respectively.

Extraction and characterization of fungal taxol

Five fungal endophytes with positive results of primary screening were inoculated into 100 ml of sterilized S-7 medium, incubated at 25 °C on a shaker for 5 days (Stierle et al. 1993) to detect taxol production. These cultures were used as seed cultures for taxol production. Then, 10–20 ml of seed culture was transferred to 2-l Erlenmeyer flasks containing 500 ml of sterilized S-7 medium for each fungal isolate and incubated for 21 days in the dark as stationary cultures. After 3 weeks of incubation, the culture was filtered through four layers of cheese cloth to remove the mycelia pellets. Then, 0.2 mg Na_2CO_3 was added to the culture filtrates with frequent shaking to reduce the amount of fatty acids that might contaminate the taxol in the broths. The harvested mycelia were frozen by liquid nitrogen, then crushed thoroughly in a mortar and extracted 3 times in 10 ml of methanol. Then, the fermentation broths were extracted with three equal volumes of dichloromethane (DCM), and the methanol fractions were reconstituted with an equal volume of distilled water and portioned with dichloromethane. The DCM fractions were combined and the solvents were condensed under reduced pressure and re-dissolved with 1 ml of HPLC grade methanol. The extracts of each fungal isolate were purified by column chromatography and examined for the presence of taxol using HPLC and LC-MS. Taxol identification was carried out by injecting 20 μl of putative samples to HPLC (Acquity HPLC; Waters, USA) equipped with a reverse phase column (RP-c18 pre-packed column; Waters) and detected with online DAD (diode array detector) set at a wavelength of 232 nm. Elution was carried out in an isocratic mode with mobile phase methanol:acetonitrile:water (20:40:40 v/v) at a flow rate of 1 ml/min and run time of 30 min. The LC-MS was carried out on fungal samples using Waters Acquity triple quadrupole tandem LC-MS. Elution was in an isocratic mode with acetonitrile:water (49:51) as mobile phase. The samples in 100 % methanol were infused into the mass spectrometer through a reverse phase C18 column and separated at a flow rate of 0.3 ml/min with column temperature of 25 °C and spray voltage of 2.2 kV by the loop injection method. The MS scanning ranged from 100 to 1,000 m/z and the mass spectral fragment ions of taxol were observed.

Assay of antitumorigenic activity

The antitumorigenic activity of fungal taxol was assayed by Potato disc tumor induction assay as described by Coker et al. (2003) using *Agrobacterium tumefaciens* as the tumor-causing agent. In this assay, healthy potatoes were surface-sterilized and cylinders were cut from them using an autoclaved and flame-sterilized cork borer (10 mm). The cylinders were given a wash in sterile distilled water and 0.5-cm-thick discs were cut from them using a surgical blade. The discs were placed aseptically in Petri plates containing 15 % water agar. *Agrobacterium tumefaciens* (MTCC No. 431) grown on yeast extract media (YEM) for 48 h at 28 °C was used for inoculation. The cell suspension was centrifuged and superseded in phosphate buffer saline (PBS: 0.043 % KH_2PO_4, 0.148 % Na_2PO_4 and 0.72 % NaCl) to attain the absorbance of 0.96±0.02 at 600 nm. Paclitaxel (Sigma Chemicals) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mg/ml and then further diluted to 0.1, 0.01, and 0.001 μg/μl, respectively. Standard taxol served as positive inhibitory control. Other controls included: DMSO with phosphate-buffered saline (PBS), DMSO without bacterium, and DMSO with the bacterium. The test solutions consisted of 400 μl of the drug (Paclitaxel) or fungal extract+100 μl of sterile water+400 μl of standardized bacterial suspension. Each disc in the Petri plate was overlaid with 50 μl of the appropriate extract/water/bacterial mix, incubated at room temperature for 15–20 days, and observed regularly. After incubation time, the discs were stained with Lugol's reagent (I_2KI: 5 % I_2+10 % KI in distilled water). Stained potato discs were viewed under a dissecting microscope and tumors were counted. The experiment with fungal extracts for all 5 endophytic fungi was repeated thrice at all dilutions and the results were analyzed. Bacterial viability was determined by incubating the drug (Paclitaxel: 0.001 mg/ml) and extracting with bacterial suspension (in PBS solution) in YEM broth. After 3, 6, 9, and 12 h of inoculation, the growth was monitored by taking absorbance at 600 nm. All the experiments were performed in triplicate.

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $P<0.05$. All

the analyses were performed by using Graph Pad Prism 5.1 software.

Nucleotide sequence accession numbers

The partial sequences of the ITS nrDNA and *bapt* genes obtained from the 5 endophytic isolates were deposited in GenBank (NCBI) under the accession numbers KC924920, KF010838, KF010839, KF010840, KF010841 and KC924919, KF010842, KF010843, KF010844, KF010845, respectively.

Results

Primary screening of taxol-producing endophytic fungi

All 60 fungal isolates isolated from *T. baccata* ssp. *wallichiana* were screened for the presence of *dbat* and *bapt* genes. Eight out of 60 fungi had about 200-bp amplified fragments of the *dbat* gene. Presence of *dbat* gene is essential for taxol biosynthesis but cannot be relied on completely because some fungi having the *dbat* gene may produce baccatin III, but not taxol. Therefore, these fungi were further screened for the presence of the *bapt* gene. Five fungal isolates (TBPJ-B, TBPJ-A, TBPJ-13, B-7, and C-1) showed amplification of a 530-bp fragment of the *bapt* gene, suggesting that all of them may produce taxol. Sequences of *bapt* gene of endophytic fungi were analyzed using BLASTx and aligned with the protein sequences of the *bapt* gene of various *Taxus* species using Clustal W software (Larkin et al. 2007). The partial protein sequences of the *bapt* gene of all 5 fungi showed high homology with protein sequences of the *bapt* gene of various yew species, confirming the presence of a taxol biosynthetic pathway in all the endophytic fungal strains (Fig. 1).

Diversity of taxol-producing endophytes of *T. baccata*

The five fungal isolates selected on the basis of molecular marker screening were identified on the basis of morphological characteristics and unique phenotypic characters. The identified fungi belonged to the phylum Ascomycota. To validate the reliability of morphological and microscopic identification, all five endophytic strains were subjected to molecular identification based on ITS nrDNA sequence analysis. The phylogenetic tree reconstructed using ITS sequences of TBPJ-B, TBPJ-A, TBPJ-13, B-7, and C-1 is shown in Fig. 2. The phylogenetic tree constituted of four clades. Strain TBPJ-B located in clade 1 with a boot-strap value of 99 % was clustered with *Fusarium redolens* (KC924920), B-7 was clustered with *Fusarium tricinctum* (KF010839) in clade 2 (boot-strap support 96 %), and C-1 with *Gibberella avenacea* (KF010838) in clade 3 (boot-strap support 93 %). Similarly, TBPJ-A clustered with *Microdiplodia* sp. G16A (KF010841) with a boot-strap value of 74 % and TBPJ-13 with *Paraconiothyrium brasiliense* (KF010840), were clustered in clade 4. Strain TBPJ-13 with a low boot-strap value of 42 %.

Identification of fungal taxol

The extracts of five endophytic fungal species which showed positive results in primary screening were subjected to detection of fungal taxol by high performance liquid chromatography and

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T.xmedia (AAT73200)          PLSAIDNMARAFS---NVLLVYAANMDRVSADPAKVIREALSKVLVYYYPFAGRLRNKENGELEVECTGQGVLFLEAMADSDLSVLTDLDNYNPSFQQLI
T.mairei (AEJ84001)          PLSAIDNMARAFS---NVLLVYAANMDRVSADPAKVIREALSKVLVYYYPFAGRLRNKENGELEVECTGQGVLFLEAMADSDLSVLADLDNYNPSFQQLI
T.cuspidate (AAL92459)       PLSAIDNMARAFS---NVLLVYAANMDRVSADPAKVIREALSKVLVYYYPFAGRLRNKENGELEVECTGQGVLFLEAMADSDLSVLTDLDNYNPSFQQLI
T.sumatrana (ACN62085)       PLSAIDNMARAFS---NVLLVYTADMDRVSADPAKVIREALSKVLVYYYPFAGRLRNKEDGELEVECTGQGVLFLEAMADSDLSVLTDLDDYKPSFQQLI
G.avenacea                   PLSAIDNIARAFS---NILLVYSA-SHRVSADPAKVIREALSKVLVYYYPFAGRLRKKENGELEVECTGQGVLFVEAMADSDLSVLTDLDDYNPSFQQLL
P.brasiliense                PLSAIDNIARAFS---NILLVYSA-SHRVSADPAKVIREALSKVLVYYYPFAGRLRKKENGELEVECTGQGVLFVEAMADSDLSVLTDLDDYNPSFQQLL
F.tricinctum                 PLSAIDNIARAFS---NILLVYSA-SHRVSADPAKVIREALSKVLVYYYPFAGRLRKKENGELEVECTGQGVLFVEAMADSDLSVLTDLDDYNPSFQQLL
Microdiplodia sp.            PLSAIDNIARAFS---NILLVYSA-SHRVSADPAKVIREALSKVLVYYYPFAGRLRKKENGELEVECTGQGVLFVEAMADSDLSVLTDLDDYNPSFQQLL
F.redolens                   PLSAIDNIARAFS---NILLVYSA-SHRVSADPAKVIREALSKVLVYYYPFAGRLRKKENGELEVECTGQGVLFVEAMADSDLSVLTDLDDYNPSFQQLL
T.baccata (AGJ71919)         QLSTLDNLPGVRENIFNTLLVYNA-SDRVSADPAKVIRQALSKVLVYYSPFAGRLRKKENGDLEVECTGEGALFVEAMADTDLSVLGDLDDYSPSLEQLL
T.canadensis (ABW84235)      QLSTLDNLPGVRENIFNTLLVYNA-SDRVSADPAKVIRQALSKVLVYYSPFAGRLRKKENGDLEVECTGEGALFVEAMADTDLSVLGDLDDYSPSLEQLL
T.globosa (ABW84246)         QLSTLDNLPGVRENIFNTLLVYNA-SDRVSADPAKVIRQALSKVLVYYSPFAGRLRKKENGDLEVECTGEGALFVEAMADTDLSVLGDLDDYSPSLEQLL
                              **::**:. . .   * **** *  .***********:********* *******:**:*:*******:*.**:*****:***** ***:*.**::**:

T.xmedia (AAT73200)          FSLPQDTDIEDLHLLIVQ----VTRFTCGGFVVGANVYGSTCDAKGFGQFLQGMAEMA
T.mairei (AEJ84001)          FSLPQDTDIEDLHLLIVQ----VTRFTCGGFVVGTNVYGSVCDAKGFGQFLQGMAEMA
T.cuspidate (AAL92459)       FSLPQDTDIEDLHLLIVQ----VTRFTCGGFVVGANVYGSACDAKGFGQFLQSMAEMA
T.sumatrana (ACN62085)       FSLPQDTDIEDLHLLIVQ----VTRFTCGGFVVGANVYGSVCDAKGFGQFLQGMAEMA
G.avenacea                   FSVPQDADIEDLHLFIVQVFMQVTRFTCGGFVVGANVYGSVCDGKGFGQFLQSMAEMA
P.brasiliense                FSVPQDADIEDLHLFIVQVFMQVTRFTCGGFVVGANVYGSVCDGKGFGQFLQSMAEMA
F.tricinctum                 FSVPQDADIEDLHLFIVQVFMQVTRFTCGGFVVGANVYGSVCDGKGFGQFLQSMAEMA
Microdiplodia sp.            FSVPQDADIEDLHLFIVQVFMQVTRFTCGGFVVGANVYGSVCDGKGFGQFLQSMAEMA
F.redolens                   FSVPQDADIEDLHLFIVQVFMQVTRFTCGGFVVGANVYGSVCDGKGFGQFLQSMAEMA
T.baccata (AGJ71919)         FCLPPDTDIEDIHPLVVQ----VTRFTCGGFVVGVSFCHGICDGLGAGQFLIAMGEMA
T.canadensis (ABW84235)      FCLPPDTDIEDIHPLVVQ----VTRFTCGGFVVGVSFCHGICDGLGAGQFLIAMGEMA
T.globosa (ABW84246)         FCLPPDTDIEDVHPLVVQ----VTRFTCGGFVVGVSFCHGICDGLGAGQFLIAMGEMA
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Fig. 1 Multiple sequence alignment of the *bapt* protein of various *Taxus* species aligned with *bapt* protein of 5 endophytic fungi. NCBI accession numbers of *bapt* gene of fungal isolates are as follows: *Gibberella avenacea* (KF010843), *Paraconiothyrium brasiliense* (KF010844), *Fusarium tricinctum* (KF010842), *Microdiplodia* sp. (KF010845), and *Fusarium redolens* (KC924919)

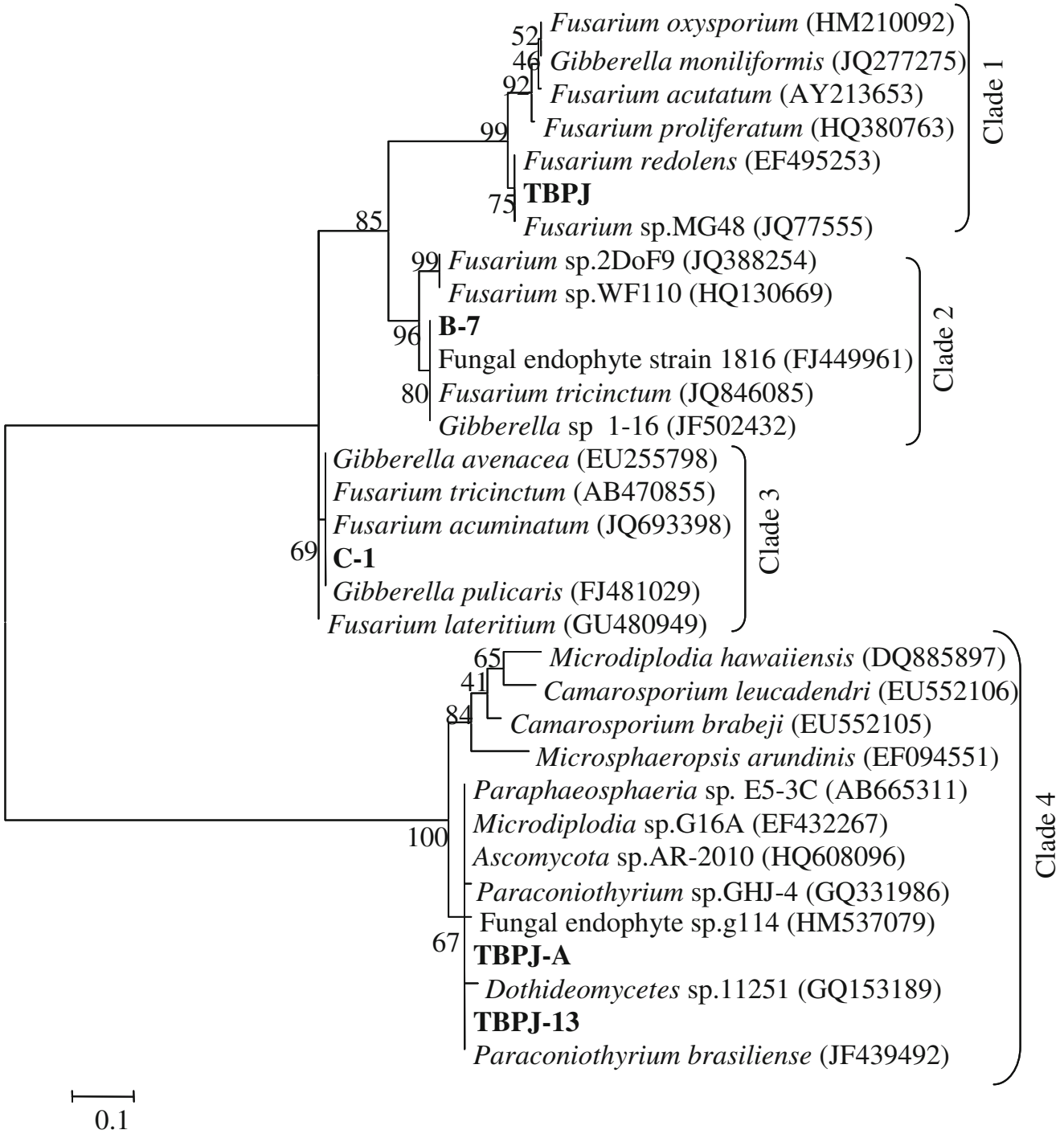

Fig. 2 Phylogenetic relationship of 5 endophytic fungi as inferred from ITS nrDNA sequence data using parsimony analysis. Numbers at nodes are bootstrap scores (above 50 %) obtained from 1,000 replications. *Scale bar* the number of nucleotide substitutions per site

mass spectroscopy. The HPLC peak of taxol from fungal extracts have the same retention time as that of standard taxol, i.e. retention time of standard taxol 3.43 min and fungal taxol 3.44 min approx. (Fig. 3). Similarly, MS spectra of authentic taxol yielded an $(M+H)^+$ peak at *m*/*z* 854.7 and an $(M+Na)^+$ peak at *m*/*z* 876.8, and the fungal taxol also yielded a peak MH^+ at *m*/*z* 854.3 and MNa^+ at *m*/*z* 876.3 with characteristic fragments at *m*/*z* 569, 551, 509, 286, and 268 (Fig. 4). The peaks of fungal taxol gave *m*/*z* ratios similar to the molecular ions of standard taxol, verifying that the 5 endophytic strains can produce taxol in vitro. Among the 5 taxol-producing fungi, TBPJ-B had the highest taxol yield of 66.25 μg/l, in comparison with those of TBPJ-A which produced 27.40 μg/l, B-7 produced 23.47 μg/l, TBPJ-13 produced 19.60 μg/l, and C-1

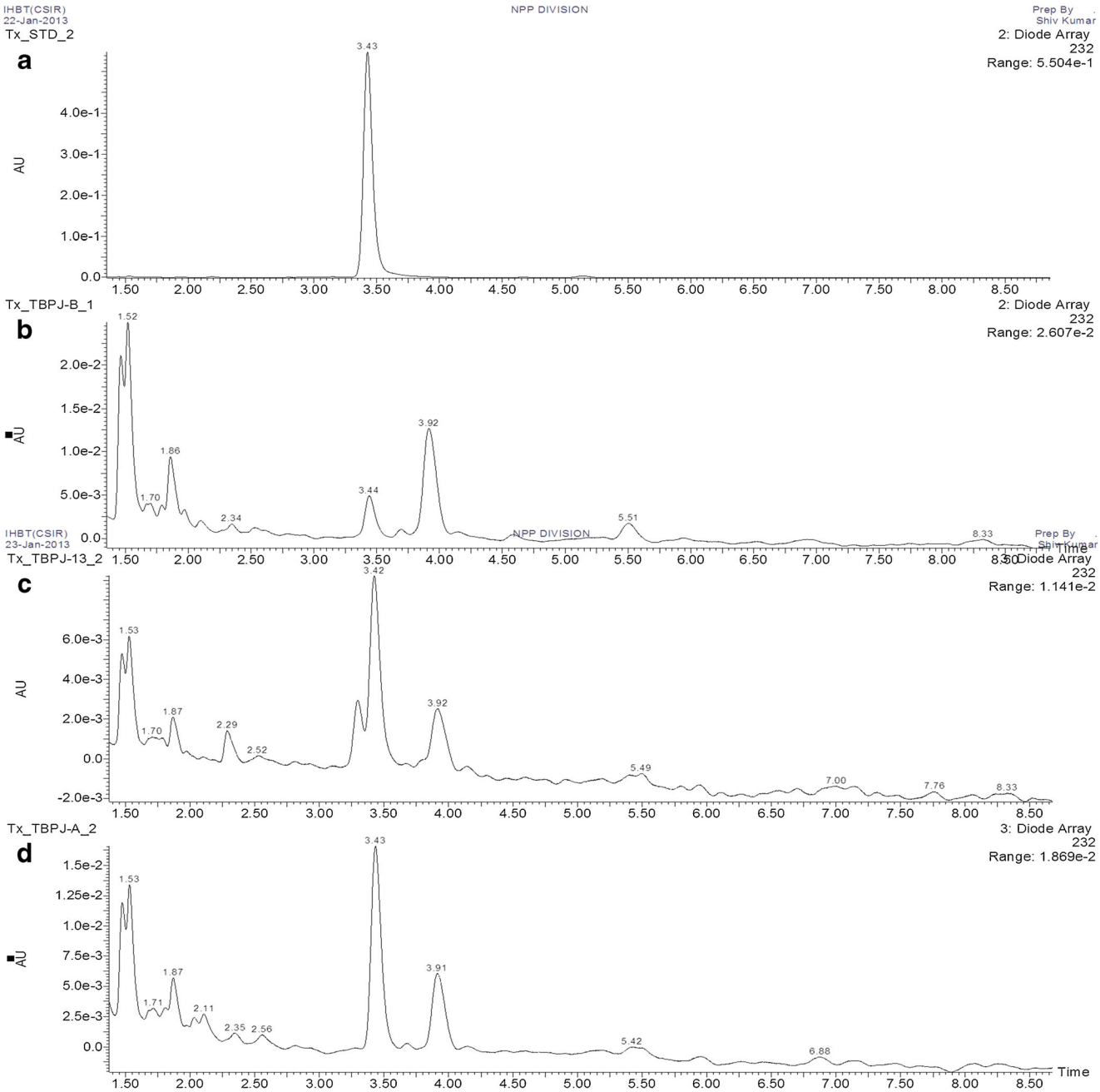

Fig. 3 Quantification and identification of taxol by HPLC. **a** Elution profile of standard taxol, **b**–**d** elution profile of taxol from the extracts of fungal isolates: **b** TBPJ-B, **c** TBPJ-13, **d** TBPJ-A

produced 11.03 μg/l of taxol in S-7 semi synthetic liquid medium.

Potato disc tumor induction assay

Agrobacterium tumefaciens (MTCC No. 431) was used as the tumor-inducing agent. Three internal control treatments were used in this study (Table 1). *A. tumefaciens* with DMSO induced at least 10 tumors per potato disc. DMSO alone did not induce any tumors. Thus, DMSO as a solvent neither interfered with the activity of the bacterium nor induced a tumor itself. Authentic taxol served as positive control and inhibited tumor production at all the tested concentrations. Fungal taxol from 5 endophytes (TBPJ-B, TBPJ-A, B-7, TBPJ-13, and C-1) also inhibited tumor formation in the same way as the authentic taxol (Fig. 5), and this was justified as starch in the potato tissue took up the stain and appeared dark brown in color, but tumors produced by *A. tumefaciens* did not take up the stain and appeared creamy to orange (Mc Laughlin and Rogers

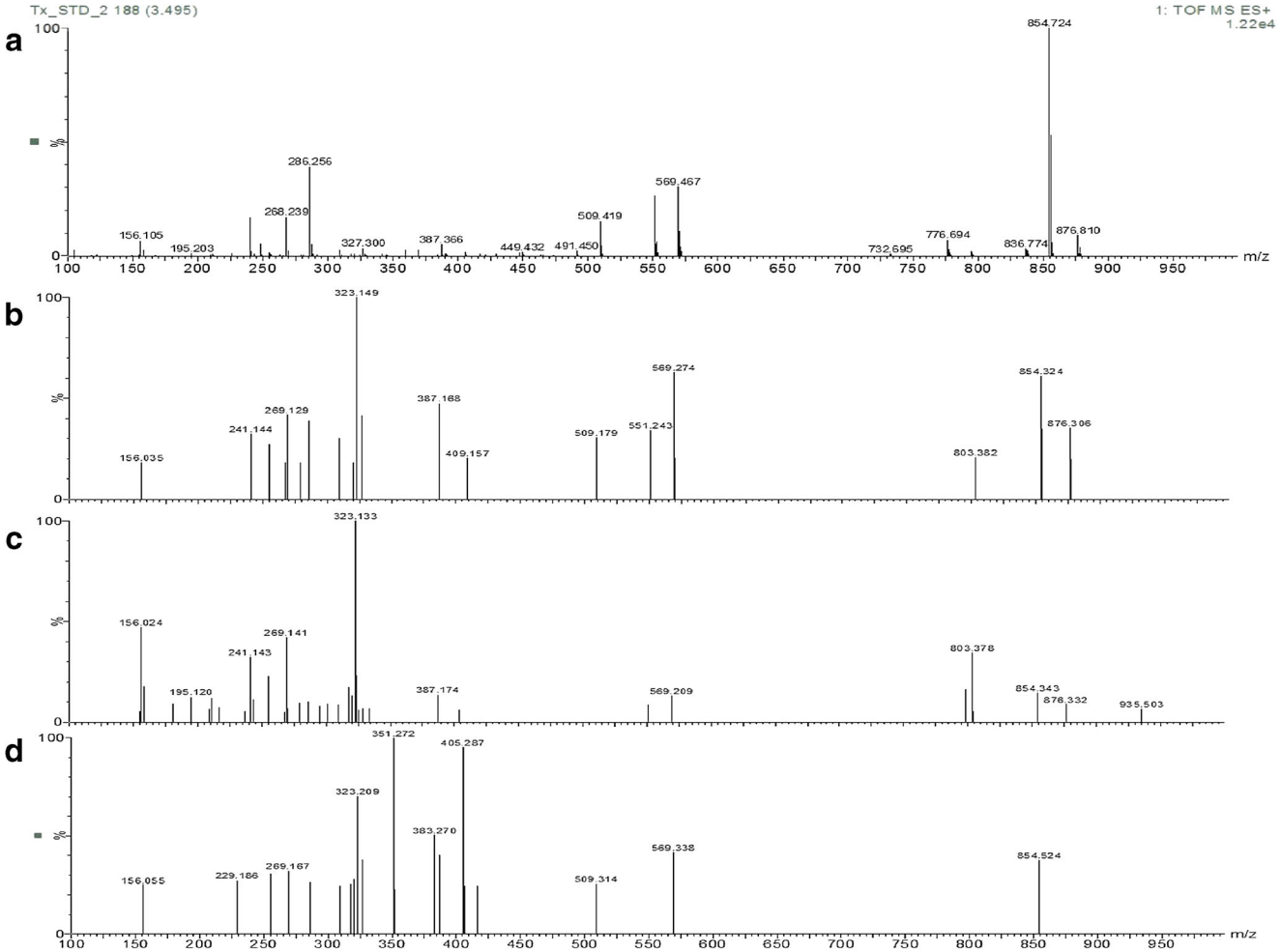

Fig. 4 Mass spectra of **a** standard taxol and taxol isolated from fungal strain, **b** TBPJ-B, **c** TBPJ-A, **d** TBPJ-13. Characteristic ions at *m/z* 854 $(M+H)^+$ and *m/z* 876 $(M+Na)^+$ were recorded along the characteristic fragmentation pattern; specific ions were recorded at *m/z* 569, 551, 509, 286, and 268 both in standard and fungal taxol

Table 1 Tumor induction (indicated + or -) by *Agrobacterium tumefaciens* at different concentrations of standard taxol and fungal extract

Potato discs treated with:	Tumors produced
Pure methanol (solvent control)	–
A. tumefaciens (tumor initiation control)	+
A. tumefaciens + DMSO	+
Taxol (1 mg/ml stock)	
(a) 0.1 μg/μl	–
(b) 0.01 μg/μl	–
(c) 0.001 μg/μl	–
TBPJ-B, TBPJ-A, TBPJ-13, B-7 & C-1	
(a) Undiluted sample	–
(b) ½ dilution	–
(c) ¼ dilution	–
(d) 1/8 dilution	–
(e) 1/16 dilution	–

– no formation of tumours; + formation of visible tumors; assay was performed in triplicate

1998). Bacterial viability tests showed that the standard drug and the drug in the fungal extracts did not affect the viability of the bacteria, i.e., the drug did not hinder bacterial tumor-causing ability (Table 2).

Discussion

Endophytic fungi capable of taxol production obtained from the bark of *Taxus baccata* ssp. *wallichiana* in the northern Himalayan regions of India represented a phylogenetically diverse array of fungal taxa, including some frequent and some rare genera, confirming that a few species are frequent colonizers and a majority of groups are rare inhabitants in woody plants of temperate to sub-tropical regions (Tejesvi et al. 2005). We selected northern Himalayan yew as the source for isolating endophytic fungi as, to date, no endophytes having taxol-generating capability have been reported from this plant growing in this region. Five taxol-producing endophytes were selected from a number of isolated endophytic

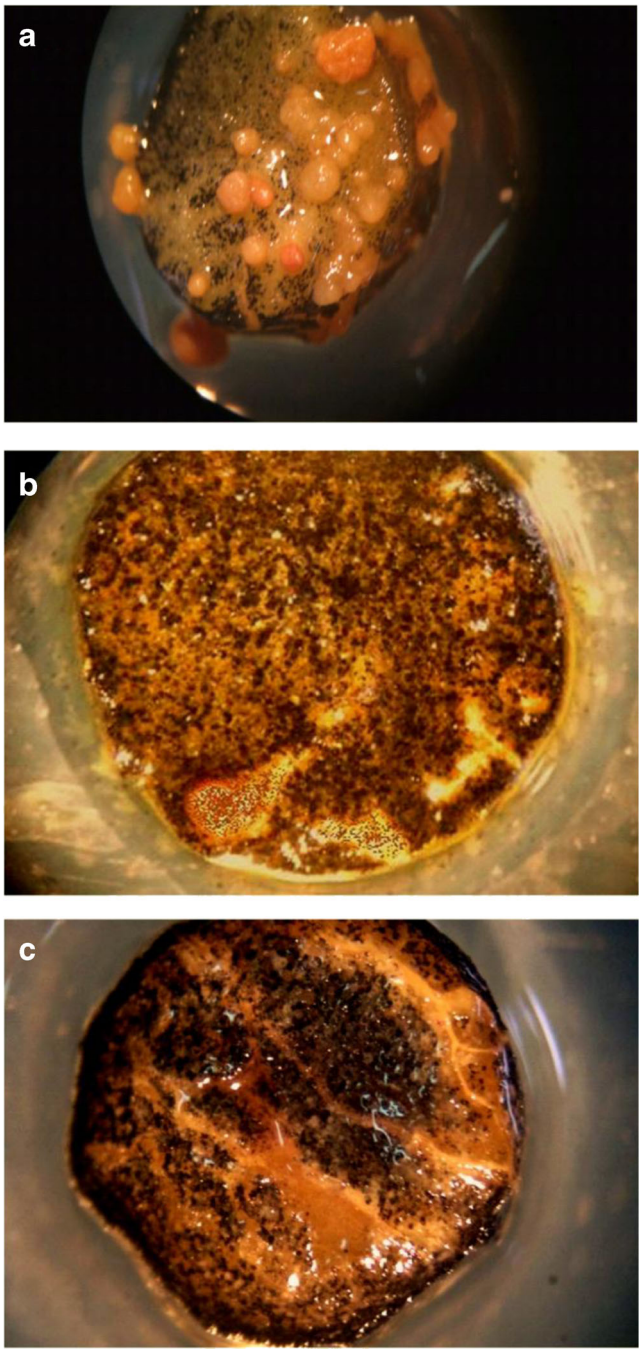

Fig. 5 Potato disc tumor induction assay. **a** Tumor formation was observed on potato disc treated only with *Agrobacterium tumefaciens*, and no tumor formation was observed on potato disc treated with **b** *A. tumefaciens* + standard taxol or **c** *A. tumefaciens* + fungal extract (5 endophytic fungi ,respectively)

fungi on the basis of molecular screening, bringing into consideration key genes involved in the biosynthesis of taxol. In contrast to the biochemical screening (traditional) method, molecular marker-based screening is a rapid, economical, and efficient alternative for the screening of taxol-producing endophytic fungi (Zhang et al. 2008; Mirjalili et al. 2012). The

Table 2 Growth of *Agrobacterium tumefaciens* on YEM medium (O.D. at 600 nm) as a function of incubation time in phosphate-buffered saline (0.001 mg/ml of Paclitaxel and undiluted fungal extract from 5 fungal isolates was used)

Exposure time (h)	Growth			
	Negative control (bacterium inoculum)	Positive control (DMSO+ bacterium)	Paclitaxel (taxol)	Fungal extract
3	0.20±0.03 d	0.16±0.031 c	0.18±0.03 c	0.16±0.02 c
6	0.33±0.03 c	0.25±0.032 c	0.25±0.04 c	0.23±0.04 c
9	0.45±0.04 b	0.40±0.025 b	0.37±0.05 b	0.35±0.03 b
12	0.58±0.07 a	0.54±0.070 a	0.53±0.06 a	0.47±0.06 a

Values sharing a common letter within the column are not significant at $P<0.05$. Values are mean±SD

main advantage with this method is that it is not dependent on the production of taxol; rather, it can indicate the presence of some key genes required for taxol biosynthesis in the fungal genome. The taxol biosynthetic pathway in yew trees involves 19 enzymatic steps (Croteau et al. 2006), and we chose *dbat* (involved in the formation of Baccatin III) and *bapt* (involved in phenylpropanoid side chain formation at C13), two main end step genes in taxol biosynthesis, for primary screening to choose taxol-producing endophytes. The use of gene-specific PCR amplification for screening the isolated endophytes made it feasible to screen all the isolates, which would have been very laborious and practically time consuming if all the isolated endophytes were to be put through biochemical screening.

Extracts of all the five selected endophytic fungi (based on primary screening) were put through chromatographic analysis (HPLC and LC-MS) for the detection of fungal taxol. *Fusarium* and some *Aspergillus* species have been reported earlier to be capable of producing taxol in vitro (Caruso et al. 2000; Zhao et al. 2008; Deng et al. 2009), but *F. redolens*, *F. tricinctum* and *G. avenacea* are the first ever reports of endophytic fungi capable of taxol production obtained from *T. baccata* ssp. *wallichiana*. *Paraconiothyrium brasiliense* and *Microdiplodia* sp. reported from other *Taxus* plants have not been acquired from the Himalayan yew until now, suggesting that yews growing in different geographical settings can harbor novel, highly diverse taxol-producing endophytic fungi, and that certain taxol-producing fungi seem to be host-specific. These endophytes that possess the capability to produce such important secondary metabolites may succeed in occupying a niche within the plant tissue or even contribute to host defence against the invading pathogens (Liu et al. 2009; Chandra 2012).

Quantitative HPLC analysis of the fungal taxol showed that TBPJ-B (*Fusarium redolens*) produced the maximum amount

of taxol compared to other isolates. These results are comparable with previously reported taxol-producing fungi isolated from different geographical settings (Stierle et al. 1993; Gangadevi and Muthumary 2008; Zhao et al. 2008, 2010). The antimitogenic activity of fungal taxol was assessed by potato disc tumor induction assay. This assay is known for its simplicity and reliability by many researchers (Coker et al. 2003) and has been used in screening of anti-tumor agents irrespective of their mode of action. Both fungal taxol and authentic taxol inhibited tumor formation in potato discs, authenticating that the fungal taxol has antitumorogenic activity. The results of the viability test in the present study depicted that the action of the drugs tested is on the formation of tumors not on the bacterial viability. In conclusion, we are reporting 5 new endophytic fungi for the first time which produce taxol from the Himalayan yew. All these fungi showed anti-mitotic activity determined by potato disc tumor induction assay. These results also suggested that PCR amplification of genes involved in taxol biosynthesis is an efficient and reliable method for pre-screening of taxol-producing fungi.

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