Taxol Production by an Endophytic Fungus, *Fusarium redolens*, Isolated from Himalayan Yew

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Different endophytic fungi isolated from Himalayan Yew plants were tested for their ability to produce taxol. The BAPT gene (C-13 phenylpropanoid side chain-CoA acetyl transferase) involved in the taxol biosynthetic pathway was used as a molecular marker to screen taxol-producing endophytic fungi. Taxol extracted from fungal strain TBPJ-B was identified by HPLC and MS analysis. Strain TBPJ-B was identified as *Fusarium redolens* based on the morphology and internal transcribed spacer region of nrDNA analysis. HPLC quantification of fungal taxol showed that *F. redolens* was capable of producing 66 µg/l of taxol in fermentation broth. The antitumour activity of the fungal taxol was tested by potato disc tumor induction assay using *Agrobacterium tumefaciens* as the tumor induction agent. The present study results showed that PCR amplification of genes involved in taxol biosynthesis is an efficient and reliable method for prescreening taxol-producing fungi. We are reporting for the first time the production of taxol by *F. redolens* from *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger. This study offers important information and a new source for the production of the important anticancer drug taxol by endophytic fungus fermentation.

**Keywords:** *Agrobacterium tumefaciens*, endophytic fungi, ITS sequence, HPLC, Northern Himalayan yew, taxol (paclitaxel)

**Introduction**

Endophytic fungi reside in living plant tissues, apparently without inflicting negative effects, and have been found in all the species of plants studied to date. Some endophytic fungi are known to improve the ecological adaptability of its hosts [15, 18, 19], and certain endophytes are capable of synthesizing the medicinal products produced in plants [23]. Presently, much work has been focused on the isolation of endophytic fungi from medicinal plants, discovering many undescribed endophytic fungal species, some of which have potential to be used in the production of medicines [5, 6, 7, 8, 12, 31, 35]. Stierle *et al.* [20] obtained the first taxol-producing fungi, *Taxomyces andreanae*, from the Pacific Yew *Taxus brevifolia*, which paved the way for utilizing this novel and promising approach for production of this valuable compound. Since then, extensive research searching for endophytic fungi from *Taxus* plants in different geographical settings have led to the discovery of some important taxol-producing fungi with taxol yields ranging from 24 ng/l to 187 µg/l [6, 20, 34, 36]. The need for the search of taxol-producing endophytic fungi came into consideration, as this compound was found to exist in low concentrations of 0.01%–0.05% from the most common source, the bark of trees belonging to the *Taxus* family [16, 29]. Furthermore, *Taxus* trees are rare and slow growing, and the traditional method of extracting taxol requires processing of large amounts of bark, causing irreplensishable damage and loss of the endangered natural source, and even the yield of pure drug is low. Although the amount of taxol found in most of the *Taxus*-associated endophytic fungi is small compared with that of trees, the short generation time and high growth rate of fungi make it worthwhile to investigate these species for taxol production [13].

Gene coding for taxadiene synthase (TS), a rate limiting enzyme in the taxol biosynthetic pathway, were used as a molecular marker to screen *Taxus*-associated fungi for taxol
production [37]. Zhang et al. [33] have used the genes coding for 10-deacetyl/baccatin III-10-O-acetyl transferase (DBAT) and C-13 phenylpropanoid side chain-CoA acyltransferase (BAPT) as molecular markers for screening taxol-producing endophytic fungi. DBAT catalyzes the formation of baccatin III, which is the immediate diterpenoid precursor of taxol [25], and BAPT catalyzes the selective 13-O-acylation of baccatin III, with β-phenylalanoyl-CoA as the acyl donor, to form N-debenzoyl-2′-deoxycodexin, that is, it catalyzes the attachment of the biologically important taxol side chain precursor [26].

Taxus baccata L. subsp. wallichiana (Zucc.) Pilger, also known as Himalayan Yew, is the only species of Taxus that is found in the temperate Himalayas at an altitude of 1,800–3,300 m amsl. This is a relatively young, non-resinous, evergreen conifer that undergoes cross-pollination and has been found to grow best in well drained moist areas, in cool, temperate to subtropical climates. To the best of our knowledge, no work for isolation of taxol-producing endophytic fungi from *T. baccata* subsp. *wallichiana* growing in the northern Himalayan region of India has been reported to date.

In this study, we are reporting a new taxol-producing endophytic fungus isolated from the bark of the Himalayan yew. The taxol synthesizing ability of this fungus was confirmed by BAPT sequence analysis. The presence of taxol in the fungal broth was determined by reverse-phase HPLC and mass spectroscopy. The fungus was identified as *Fusarium redolens* based on the morphological characters and internal transcribed spacer (ITS) region of nrDNA sequence analysis. The antimiotic activity of fungal taxol was confirmed by inhibition of tumors in potato disc tissue using *Agrobacterium tumefaciens* as the tumor inducing agent.

**Materials and Methods**

**Isolation of Endophytic Fungi**

Bark samples (1 × 3 cm) were harvested from the stem of relatively young *T. baccata* subsp. *wallichiana* from Bhadrewah (district Doda, Jammu & Kashmir, India) and were placed in a sealed plastic bag, transported to the laboratory, and stored at 4°C. For isolation of endophytic fungi, bark pieces were washed thoroughly under running tap water, followed by sterile distilled water. Bark pieces were then surface-sterilized by immersing in 70% aqueous ethyl alcohol (v/v) for 60 sec to kill epiphytic microorganisms, followed by washing in 4% sodium hypochlorite for 60–90 sec. Finally, the bark pieces were rinsed several times in sterile distilled water. The excess moisture on the bark surface was blotted using sterile filter paper. Using a flame-sterilized sharp blade, the layers of outer bark from the surface-sterilized bark pieces were removed systematically. Small pieces of inner bark (~ 0.5 × 0.5 × 0.5 cm) were cut and then plated carefully on the surface of potato dextrose agar (PDA) medium supplemented with ampicillin (50 μg/ml) in Petri plates and sealed. The Petri plates were incubated at 25°C–28°C for 5–10 days and were checked regularly for the growth of endophytic fungal colonies. Pure isolates were obtained by picking individual fungal hyphal tips from the PDA plates and placing on fresh PDA medium and incubating at 25°C for 10 days. Each fungal culture was carefully checked for purity and transferred to another PDA plate by the hyphal tip method [22].

**Screening for Taxol-Producing Endophytic Fungi by PCR**

Isolated fungal samples were inoculated aseptically and individually in 20 ml of potato dextrose broth in 150 ml Erlenmeyer flasks. All cultures were incubated at 25°C at 120 rpm for 3–5 days, and the mycelia were harvested by centrifugation (12,000 × g/10 min). Mycelia were ground using a sterile mortar-pastele into fine powder in liquid nitrogen. Genomic DNA was extracted by the CTAB method [32]. The concentration and purity of all extracted DNA were measured using a nanodrop assay. Taxol-producing fungi were screened by amplifying the *DBAT* and *BAPT* genes. The primers used to amplify the *DBAT* gene (dbat-F 5′-GGGGAGGTGCTCTCTTGGTG-3′ and dbat-R 5′-GTTACCTGAACCACCAGAGG-3′) and the *BAPT* gene (bapt-F 5′-CCTCTCTCCGCCATTGACA-3′ and bapt-R 5′-TCGCCATCTCGGATACTCTT-3′) were as described by Li et al. [11]. Thermalcycles were performed in a GeneAmp PCR system 2700 (Applied Biosystems, USA). First, the fungal isolates were screened for the presence of the *DBAT* gene. Amplification was done using dbat-F and dbat-R primers in a 20 μl PCR mixture. The PCR programme consisted of the following primer extension conditions: initial pre-heating at 95°C for 6 min; 35 cycles of 94°C for 50 sec, 50°C for 30 sec, and 68°C for 50 sec; and additionally 68°C for 10 min. The amplified DNA fragments were analyzed by agarose gel electrophoresis, and fungi showing amplification for *DBAT* were further subjected to *BAPT* gene amplification. PCR amplification was done using bapt-F and bapt-R primers in a 20 μl reaction mixture, with the following primer extension conditions: 6 min at 95°C; 35 cycles of 94°C (50 sec), 55°C (50 sec), and 68°C (50 sec); and a final extension at 68°C for 10 min. The amplified DNA fragments were analyzed by agarose gel electrophoresis. The PCR-amplified product was purified using a QiAquick PCR purification kit (Qiagen) and then cloned in *E. coli* using the pTZ 57R/T vector. Transformed colonies were carefully picked and the insert was sequenced. The *BAPT* gene sequence of endophytic strain TBPJ-B was compared by using BLASTx and aligned with the protein sequences of the *BAPT* gene of various *Taxus* species using Clustal W software [24].

**Identification of Endophytic Fungus**

The endophytic strain TBPJ-B was characterized based on its morphological characters, such as fungal culture colony, spore, and the reproductive structures [1, 28]. The isolated DNA was used as
a template to amplify the ITS regions together with the 5.8S rRNA gene through PCR, using universal primers ITS1 (5'-TCCGTAGGT GAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTATTGATATGC-3') [30], as described in Pandey et al. [17]. The sequence obtained was annotated using Sequin software and submitted to the NCBI GenBank database. A BLAST search was performed to find the possible homologous sequences of newly sequenced taxa from the GenBank database. Sequences retrieved from GenBank were added to the alignments. The phylogenetic tree was reconstructed using the maximum parsimony method and the Kimura two-parameter distance calculation by the MEGA5 software [9]. The bootstrap was 1,000 replications to assess the reliable level to the nodes of the tree.

**Extraction and Characterization of Fungal Taxol**

Pieces of agar block containing the mycelia mats from a 7–10-day-old culture plate of TBPJ-B were transferred to 100 ml of sterilized S-7 medium [20] and incubated at 25–28°C on a rotary shaker for 5 days. This culture was used as seed culture for taxol production, where 10–20 ml of seed culture was transferred to a 2 L Erlenmeyer flask containing 500 ml of sterilized S-7 medium and incubated at 25–28°C for 21 days as the stationary culture. After 3 weeks of incubation, the entire 500 ml of culture was filtered through four layers of cheese cloth to remove the mycelia. To reduce the amount of fatty acids that may contaminate taxol in culture, Na2CO3 (0.125 g) was added to the culture filtrate with frequent shaking. The harvested mycelia were dried overnight at 35–40°C. Dried mycelia were crushed and extracted in 10 ml of methanol three times and the extracts were filtered through glass wool. Then, the culture broth filtrate was extracted with three equal volumes of dichloromethane (DCM); the methanol (MeOH) fraction was reconstituted with an equal volume of distilled water and then portioned with DCM. The DCM fractions (organic phase) were collected and combined, and the solvent was then evaporated under reduced pressure at 35°C. The residue was re-dissolved in chloroform for further chromatographic analysis. Column chromatography was carried out on a glass column packed with silica gel to a 12 cm bed volume (Silica gel 60–120 mesh; Merck). The column was equilibrated in chloroform (CHCl3) and rinsed several times before loading the sample. Sample previously dissolved and stored in CHCl3, was loaded on the column and again rinsed 2–3 times with chloroform. Taxanes were eluted in 20 ml of acetone and the elute was dried in vacuo and finally dissolved in 1 ml of HPLC-grade MeOH for TLC, HPLC, and LC-MS analyses.

Thin layer chromatography (TLC) analysis was carried out on a 0.5 mm (20 x 20 cm) silica gel (lab prepared) preparative TLC plate (Silica gel GF 254; Merck). The methanolic extract from TBPJ-B as well as the methanolic solution of Paclitaxel standard (Paclitaxel Taxus brevifolia; Sigma Chemicals Co., St. Louis, USA) were chromatographed on the TLC plate, and developed in chloroform-methanol 7:1 (v/v) (105:15). A spray reagent that consisted of 1% vanillin (w/v) in sulfuric acid was used to detect the compound of interest on the TLC plate [2, 27]. Another TLC plate loaded with the same fungal sample and authentic taxol was also exposed to iodine-potassium iodide fumes in a glass chamber for confirming the presence of the compound. The fungal taxol was identified by comparison with authentic taxol, on the basis of its chromatographic mobility and Rf values. The TLC plates were also visualized under short-wave UV light at 250–270 nm to visualize the bands of desired compound. After TLC, the area of plate containing crude compound was carefully removed by scraping off silica at the appropriate Rf and eluting it with pure methanol.

After elution, the putative taxol was subjected to qualitative and quantitative analyses using HPLC (Perkin Elmer Series 200, USA). Quantification of taxol was carried out by injecting 20 µl of purified sample to the HPLC analysis, equipped with a reverse phase C-18 column (Brownlee Columns, Lichrospher 100 RP-18e pre-packed column; Perkin Elmer, USA) and detected with a DAD (diode array detector) detector at a wavelength of 232 nm. Taxol was eluted in an isocratic mode with the mobile phase (MeOH:AcN:H2O) methanol-acetonitrile-water (20:40:40 (v/v)) at a flow rate of 1 ml/min and run time of 30 min.

The fungal taxol was confirmed with a Waters Acquity triple quadrupole tandem LC-MS (Waters, USA). The HPLC portion was run isocratically with acetonitrile-water (49:51) as the 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.

**Antitumorogenic Activity Assay**

The antimitotic activity of fungal taxol was assayed by potato disc tumor induction as described in Coker et al. [3] using Agrobacterium tumefaciens as the tumor causing agent. Briefly, healthy potatoes were surface sterilized, and using an autoclaved flame-sterilized cork borer (10 mm), cylinders were cut from the potato. The cylinders were given a final wash in sterile distilled water, and 0.5 cm thick discs were cut from the cylinders using a flame-sterilized blade. The discs were placed aseptically in Petri plates containing 15% water agar. Agrobacterium tumefaciens grown on yeast extract medium (YEM) for 48 h at 28°C was used for inoculation. The cell suspension was centrifuged and suspended in phosphate buffer saline (PBS: 0.043% KH2PO4, 0.146% Na2PO4, 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 µl of the drug (Paclitaxel) or 0.1, 0.01, and 0.001 µg/µl, respectively. Standard taxol served as positive inhibitory control. Other controls included DMSO with 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 bacterium suspension. Each disc in the petriplate was overlaid with 50 µl of the appropriate extract/water/bacterial mix, incubated...
at room temperature for 15–20 days, and observed regularly. After the incubation time, the discs were stained with Lugol’s reagent (I₂KI: 5 % I₂ + 10% KI in distilled water). Stained potato discs were viewed under a dissecting microscope and tumors were counted. The experiment with the fungal extract was repeated three times at all dilutions and the results were analyzed for the formation of tumors.

Bacterial viability was determined by incubating the drug (Paclitaxel: 0.001 mg/ml) and extract with bacterial suspension (in PBS solution) in YEM broth. After 3, 6, 9, and 12 h of inoculation, the growth was monitored by taking the absorbance at 600 nm. All the experiments were performed in triplicates.

**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.

**Results**

**Isolation and Screening of Endophytic Fungi**

A total of 25 endophytic fungi harboring in the bark of the *T. baccata* subsp. *wallichiana* tree were isolated and screened for the presence of the DBAT gene. Five out of the 25 fungi had about 200 bp amplified fragments of DBAT. The presence of the DBAT gene is essential for taxol biosynthesis but cannot be relied on completely because some fungi having DBAT gene may produce baccatin III, but not taxol. Therefore, these five fungi harboring the DBAT gene were screened for the presence of the BAPT gene. All isolates showed amplification of the 570 bp fragment of BAPT. Among these, fungal strain TBPJ-B was selected for further studies based on the amount of taxol production. The sequence of BAPT obtained from TBPJ-B was deposited at NCBI under the accession number KC924919. BLASTx analysis revealed that the BAPT gene of TBPJ-B showed high homology with protein sequences of the BAPT gene of various yew plant species (Fig. 1).

**Fungal Morphology and Molecular Phylogenetics**

TBPJ-B grew rapidly when cultivated on PDA medium at 28°C for 7 days. The colonial morphological traits of TBPJ-B at the time of observation were of a pink center surrounded by white-colored margins, cottony appearance, nearly round margins, and broken edges. The culture also had some unique fragrance when the culture plate was opened. Microscopic morphologies indicated the presence of tubular, thick walled, septate hyphae, simple or branched, and sickle-shaped macroconidia and oval microconidia. Microconidia were sparse, one-celled, smooth, ovoid, non-septate, present solitary, and measured 1.75–2.5 µm in length and 1.0–1.5 µm in width. Macroconidia borne on branched conidiophores were abundant, thin walled, one to three-septate, and curved to sickle-shaped. The apical cells were elongated, and blunt to conical, and basal cells were blunt to non-notched. Three-septate conidia measured 20–35 µm × 3.75 µm and two-septate ones measured 17.2–28.8 µm × 3.5 µm. Two-septate spores were more abundant and common. Chlamydospores were not present. Based on the morphology of the fungal mycelia and characteristics of the spores, the endophytic fungus TBPJ-B seemed to belong to *Fusarium* species. BLAST analysis revealed that the ITS sequence of TBPJ-B showed 95% similarity with *Fusarium redolens*. Phylogenetic analysis also clustered TBPJ-B with *Fusarium redolens* species (Fig. 2). The ITS obtained from NCBI accession numbers are in parentheses.
Taxol Production by *Fusarium redolens*

TBPJ-B was deposited at NCBI under the accession number KC924920.

**Taxol from *Fusarium redolens***

The extracted compound from *F. redolens* in S-7 liquid medium was subjected to TLC and HPLC analysis. The presence and purity of the isolated compound from fungus was checked on TLC plate. Fungal taxol on the TLC plate, after reacting with the spray reagent on gentle heating, appeared as a bluish spot, which turned grey after 24 h, and exhibited identical Rf values as authentic taxol (Rf value of 0.36). The active principle on development of the thin layer plate appeared as a dark band under UV illuminator at 254 nm. HPLC analysis of the fungal extract and authentic taxol gave a single peak under the same conditions when eluted from a reverse-phase C18 column. The fungal extract had almost the same retention time as standard taxol, of 17.69 min (retention time of TBPJ-B taxol: 17.53 min). Similarly, in MS confirmation, the authentic taxol yielded (M+H)\(^+\) at m/z 854.7 and (M+Na)\(^+\) at m/z 876.8. The fungal taxol yielded a peak MH\(^+\) at m/z 854.3 and MNa\(^+\) at m/z 876.3 with characteristic fragment peaks at 569, 551, 509, 286, and 268 (Fig. 3). On the basis of HPLC-MS analysis endophytic fungus *F. redolens* did produce taxol. The fungal extract sample in HPLC-grade methanol, obtained after processing culture filtrate and mycelium from 21-day-old culture in S-7 semisynthetic medium, was subjected to HPLC to quantify the amount of taxol produced by the fungus. Thus, 20 μl of purified extract was injected for HPLC analysis and the peak was detected at a wavelength of 232 nm. The standard curve prepared by using peak values of different concentrations of authentic taxol served as the basis for quantification of the taxol produced by the endophytic fungi. The amount of taxol produced by *F. redolens* was found to be 66 μg/l.

**Fig. 2.** Phylogenetic relationship of *Fusarium redolens* and other *Fusarium* spp. as inferred from ITS nrDNA sequence data using parsimony analysis. Numbers at nodes are bootstrap scores (above 50%) obtained from 1,000 replications. *Neurospora crassa* was used as an out-group. The scale bar indicates the number of nucleotide substitutions per site.

**Fig. 3.** Mass spectra of (A) taxol extracted from fungal strain TBPJ-B and (B) standard taxol.
Table 1. Tumor induction (indicated + or –) by *Agrobacterium tumefaciens* at different concentrations of standard taxol and fungal extract.

<table>
<thead>
<tr>
<th>Potato discs treated with</th>
<th>Tumors produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure methanol (Solvent control)</td>
<td>–</td>
</tr>
<tr>
<td><em>A. tumefaciens</em> (tumor initiation control)</td>
<td>+</td>
</tr>
<tr>
<td><em>A. tumefaciens</em> + DMSO</td>
<td>+</td>
</tr>
<tr>
<td>Taxol (1 mg/ml stock)</td>
<td></td>
</tr>
<tr>
<td>(a) 0.1 μg/μl</td>
<td>–</td>
</tr>
<tr>
<td>(b) 0.01 μg/μl</td>
<td>–</td>
</tr>
<tr>
<td>(c) 0.001 μg/μl</td>
<td>–</td>
</tr>
<tr>
<td><em>Fusarium redolens</em> (TBPJ-B)</td>
<td></td>
</tr>
<tr>
<td>(a) Undiluted sample</td>
<td>–</td>
</tr>
<tr>
<td>(b) ½ dilution</td>
<td>–</td>
</tr>
<tr>
<td>(c) ¼ dilution</td>
<td>–</td>
</tr>
<tr>
<td>(d) 1/8 dilution</td>
<td>–</td>
</tr>
<tr>
<td>(e) 1/16 dilution</td>
<td>–</td>
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</tbody>
</table>

*–* indicates no formation of tumors; ‘+’ indicates formation of visible tumors; the assay was performed in triplicates.

**Potato Disc Tumor Induction Assay**

*Agrobacterium tumefaciens* (MTCC No. 431) was used as the tumor inducing agent. Three internal control treatments were used in our study (Table 1). *A. tumefaciens* with DMSO induced at least 10 tumors per potato disc. DMSO alone did not induce any tumor. Thus, DMSO as a solvent neither interfered with the activity of the bacterium nor induced tumor itself. Authentic taxol served as the positive control and inhibited tumor production at all the concentrations tested (*i.e.*, 0.1, 0.01, and 0.001 μg/μl). Fungal taxol also inhibited tumor formation the same way as the authentic taxol did (Fig. 4), and it 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 [14].

![Fig. 4. Potato disc tumor induction assay.](image)

(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.

Bacterial viability test showed that the standard drug and the drug in the extract did not affect the viability of the bacterium (*i.e.*, drug did not hinder the bacterial tumor-causing ability) (Table 2).

Table 2. Growth of *Agrobacterium tumefaciens* on YEM medium (OD at 600 nm) as a function of incubation time in phosphate buffered saline (0.001 mg/ml of paclitaxel and undiluted fungal extract used).

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative control (Bacterium inoculum)</td>
</tr>
<tr>
<td>3</td>
<td>0.20 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>0.33 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>0.45 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>0.58 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values sharing a common letter within a column are not significant at P < 0.05; values are mean ± SD (n = 3).
Discussion

_Fusarium redolens_, an endophyte of _T. baccata_ subsp. _wallichiana_ (Himalayan yew), may have certain biological links with its host for attaining the capability of producing taxol. This yew species ranges widely in the temperate Himalayan region and in the hills of Meghalaya, Nagaland and Manipur and most of its growth area is in moist and damp environments. By the endosymbiotic nature, several endophytic fungi may also inherit taxol-producing capability and this may be considered as a reason for the transfer of genes involved in taxol biosynthesis to endophytes harboring in different parts of yew trees.

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 _T. baccata_ subsp. _wallichiana_ growing in this region. Moreover, taxol, which holds many properties such as stability, less side-effects, and higher effectiveness, has been always in surplus demand in the market owing to its broad spectrum of antitumor action. Thus, to fulfill the increasing demand of pure drug, scientists have always been searching for efficient endophytic strains from _Taxus_ sp. and species other than _Taxus_ that have the capability of generating a good amount of taxol, and our present work is one more step towards this quest. There are several reports in the literature about the number of species and genera of endophytic fungi capable of producing taxol, including _Fusarium_ sp. [36]. However, the endophytic fungus _F. redolens_ from _Taxus_ has never been reported earlier. Our present study is the first report for the isolation, characterization, and identification of _F. redolens_ from _T. baccata_ subsp. _wallichiana_ in India that is able to generate about 66 µg/1 of taxol in semisynthetic liquid medium. The amount of taxol production by _Fusarium_ spp. reported in the literature varied from 0.13 to 286 µg/1 with strain improvement procedures [4, 5, 8, 21]. The taxol-generating endophyte in the present study was selected from a number of isolated endophytic fungi of _T. baccata_ subsp. _wallichiana_ on the basis of molecular screening, bringing into consideration key genes involved in the biosynthesis of taxol (DBAT and BAPT genes). Use of gene-specific PCR amplification for screening the isolated endophytes from the Himalayan yew bark made it feasible to scan all the isolates and choose the endophyte harboring those genes. This rapid and economic method made it possible not to screen all the isolated endophytes through biochemical analysis (TLC, HPLC, and MS) for the production of taxol, which would have been very laborious and practically time consuming.

To validate the presence of the BAPT gene in _F. redolens_, the cloning and characterization of fungal BAPT gene was also done, which resulted in partial protein sequence similarity of the fungal gene with protein sequences of the BAPT gene of various yew tree species. The presence of such an important gene of taxol biosynthetic pathway in the fungal endophyte has opened doors for increase in the yield of taxol in future, by manipulations at the genetic level. The discovery of this taxol-generating endophytic fungus having very close association with the host yew tree might help us understand the evolutionary aspect of gene transfer between the host and its endosymbiont. The combined result of the classical morphological identification with that of the molecular biological analysis in the present study clearly determined that TBPJ-B represents a new strain of taxol-generating endophytic fungi, _F. redolens_. In the present work, the extract of fungal broth was examined by chromatographic analysis (TLC, HPLC, and MS). The antitumor activity of the fungal taxol was assessed by potato disc tumor induction assay. This assay is known for its simplicity and reliability by many researchers [3] and has been used in the screening of antitumor agents, irrespective of their mode of action. Both compounds (fungal taxol and authentic taxol) inhibited tumor formation in potato discs, authenticating that the fungal taxol has antitumorigenic 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 the bacterium viability.

In conclusion, PCR amplification of genes involved in taxol biosynthesis is an efficient and reliable method for prescreening taxol-producing fungi. We are reporting for the first time the production of taxol by _F. redolens_ from _T. baccata_ subsp. _wallichiana_. This study offers important information and a new source for the production of the important anticancer drug taxol by endophytic fungus fermentation. Further studies are required to optimize the culture conditions to produce the maximum amount of taxol by this fungus.

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