

Molecular and Morphological Characterization of a Taxol-Producing Endophytic Fungus, *Gliocladium* sp., from *Taxus baccata*

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(Received July 31, 2011. Accepted August 12, 2011)

The endophytic fungal populations of different tissues of *Taxus baccata* grown at high altitudes in West Bengal, India were explored. These isolated fungal populations represented different genera, which were screened for taxol production using immunoassay technique. The culture AAT-TS-4, that produced taxol was identified as *Gliocladium* sp. based on its cultural, morphological characteristics, internal transcribed spacer, and 18S rRNA sequence analysis. Kinetics of taxol production as a function of culture growth were investigated.

KEYWORDS : Endophytes, Internal transcribed spacer region, 18S rRNA

It has been previously shown that endophytic microbial populations can easily adapt their physiology in order to establish themselves in plant host tissues. These physiological changes can result in the production of useful metabolites, which could be exploited for human use. Taxol is one such metabolite that was first isolated from the bark of Western yew, *Taxus brevifolia* [1], and later from other geographically diverse *Taxus* species. This anti-microtubule drug is used for the treatment of a broad range of human tumors, including ovarian and metastatic breast cancers [2]. Apart from cancer treatment, its application to the treatment of rheumatoid arthritis, malaria, Alzheimer's disease, and autosomal dominant polycystic kidney disease [2] has also been reported.

Using *Taxus* sp. for taxol production is ecologically unsuitable, as it requires mature trees to be sacrificed. Over the past few years, other renewable sources for the commercial scale-up of taxol production have been investigated, such as isolation from needles (leaves) [3], *in vitro* culturing of *Taxus* species [4], and synthesis from readily available 10-deacetylbaaccatin III (10DAB III) [5], but none could meet the high demand for taxol production. A novel method for the production of taxol by a cheaper industrial fermentation method was recently reported based on the discovery of endophytic fungi belonging to different diverse genera that produce taxol. Apart from fungi, some bacteria [6] and actinomycetes [7]

that produce taxol have also been discovered. As India has a large wealth of medicinal plants containing an abundance of *Taxus baccata*, a screening programme was initiated to isolate endophytic microorganisms from *T. baccata* for production of taxol. Accordingly, this study focused on the screening of endophytic fungi for taxol production and the identification of industrially important fungi based on their cultural, morphological, and molecular characteristics. Our previous studies showed that this fungus produces taxol as well as its precursor 10DAB III [8]. Based on its morphological and molecular characteristics, we identified the fungus as *Gliocladium* sp. Some of the species of this genus have been identified as mycoparasites, and many novel secondary metabolites have been discovered from different species [9, 10]. However, this is the first report of fungus *Gliocladium* sp. isolated as an endophyte of *T. baccata*, which produces taxol.

Materials and Methods

Isolation of endophytic fungi from *T. baccata*. Endophytic fungi were isolated from the bark, stem, and needles of *T. baccata* obtained from West Bengal, India. The samples were cut into small pieces (approximately 0.5 × 0.5 cm), surface-sterilized with 0.01% mercuric chloride (HgCl₂) solution for 1 min, and washed thoroughly with sterile distilled water [11-13]. The outer bark was teased apart

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with the help of a sterilized sharp blade in order to obtain the inner bark (stem). Residual water on the sample surface was removed by soaking on sterile blotting paper. Small pieces of stem and needles were placed on the surface of potato dextrose agar (PDA). After 10~15 days, fungi were observed growing from the stem and needle fragments on the plates. Individual hyphal tips of the various fungi were then transferred from the PDA plates, placed again on the new PDA plates, and incubated at room temperature for at least 10~15 days. Each fungal culture was checked for purity and transferred to agar slants by the hyphal tip and single spore isolation methods [13, 14]. Of the fungal population, only slow growing and unusual fungi were considered for further study. Stock cultures were maintained by subculturing at monthly intervals. After growing at pH 7 and 25°C for 7 days, the slants were maintained at 15°C. From an actively growing stock culture, sub-cultures were made on fresh slants. After 7 days of incubation at pH 7 and 25°C, they were used as the starting material for the fermentation experiments.

Screening of endophytic fungi for taxol production.

Production of taxol by the 40 endophytic fungi isolated from different plant parts of *T. baccata* was studied by a two-stage fermentation procedure. In the first stage, these fungi were grown in submerged culture, whereas in the second stage, they were grown as a stationary culture. These fungi were grown in 500 mL Erlenmeyer flasks containing 100 mL of modified mycological medium [15]. The flasks were inoculated with agar blocks containing mycelium from 7-day-old slants. The inoculated flasks were incubated at 25~27°C on a rotary shaker (240 rpm) for 5 days. These cultures were then used as seed cultures (first stage). For taxol production, 10 mL seed cultures were transferred to 500 mL flasks containing 100 mL of modified S7 medium [15]. The flasks were incubated at 25~27°C for 21 days as a stationary culture (second stage). The culture was then harvested and passed through four layers of muslin cloth to separate the mycelial mat from the culture filtrate 3 wk after the inoculations. Both culture filtrate and mycelia were lyophilized to dryness, followed by extraction three times with equal volumes of chloroform: methanol (9 : 1) each time. These extracts were then pooled and dried with anhydrous sodium sulphate and concentrated at 40°C *in vacuo* to yield crude extract. A small amount of crude extract was dissolved in methanol, diluted in sample diluting buffer (0.25% bovine serum albumin, phosphate buffered saline), and then used for screening of taxol-positive cultures using immunoassay technique competitive inhibition enzyme linked immunoassay (Hawaii biotechnology group, Hawaii), according to the procedure described by the manufacturers [16]. It was observed that most of the chloroform extracts were not completely soluble in methanol.

Production of taxol by fungal strain AAT-TS-4₁ at different time intervals.

The flasks were incubated at 27°C for different (2, 5, 10, 15, and 21 days) time intervals as a stationary culture (second stage). After 2, 5, 10, 15, and 21 days of incubation, the culture was harvested and processed. Fermentation, extraction, and quantification were carried out as described above.

Cultural and morphological characters of the fungus.

To study cultural and morphological characteristics, the fungus was grown on PDA. Cultural characteristics such as color and colony surface texture were determined by visual observation. Other characteristics of the fungus like mycelia, conidiophores, and conidia were studied microscopically (Axiovert 25 Inverted microscope; Carl Zeiss, Jena, Germany; Nikon Eclipse E200; Nikon, Tokyo, Japan).

Nutritional studies and factors affecting growth and sporulation of the fungus.

Fungus was grown on the following natural and semisynthetic media: PDA, oat meal agar (OMA), corn meal agar (CMA), and V-8 juice agar prepared as described by the manufacturer (Himedia, Mumbai, India). To study growth of the fungus on solid media, a mycelial disc (8 mm) cut from a sporulating 20-day-old culture grown on PDA was inoculated in the centre of the Petri dish (10 cm diameter) and incubated at 25~27°C in a B.O.D incubator. Observations of the growth pattern and sporulation were recorded when the culture was 5 days old. Sporulation of the fungus on solid media (PDA, CMA, OMA, and V-8 juice) was determined based on the number of spores present per field under uniform magnification and categorized in the following grades: no sporulation, good, and excellent. Data set comprising the morphological characteristics based on the previously published description is in Table 1. Information regarding shape, temperature, conidiophores, morphological characteristics of the colony, and average diameter of the colony is also listed in Table 1.

Genomic DNA isolation.

Genomic DNA of the fungal culture was extracted by the salting out method [17]. For this, 5 g of fungal mycelia was ground into fine powder using liquid nitrogen and suspended in 5 mL of SET buffer (75 mM NaCl, 25 mM EDTA [pH 8.0], 20 mM Tris [pH 7.5]). About 100 µL of lysozyme (final concentration [conc.] 1 mg/mL) was added to the above suspension, which was incubated at 37°C for 1 hr, followed by addition of 140 µL of proteinase K (final conc. 0.5 mg/mL) and 600 µL of 10% SDS and incubation again for 2 hr at 55°C with occasional mixing. Sample was precipitated by the addition of 2 mL of 5 M NaCl (final conc. 1.25 M), and the mixture was cooled to 37°C. Then, 5 mL of chloroform was added and mixed for about

Table 1. Differences in morphological characteristics of fungus AAT-TS-4, growing on different media

Characters	PDA	V-8 juice	OMA	CMA
Average diameter (mm) of the colonies after 5 days	25	18	40	30
Morphological characters of the colony	Circular, smooth, flocculose and dense	Circular, smooth, flocculose and dense	Sparse, woolly with definite periphery	Circular, not so compact, cottony
Colony color	White in front and pale cream on reverse	White in front and dark cream on reverse	White in front and reverse	White in front and pale cream on reverse
Conidiophore	Erect, branched to unbranched verticillium like, with 2~5 phalides	No conidiophores seen	Erect, branched to unbranched verticillium like, with 2~5 phalides	Erect, branched to unbranched verticillium like, with 2~5 phalides
Conidia	+++	+	+++	++
Conidia shape	Cylindrical, oval, and curved	Non	Curved, oval, and cylindrical	Cylindrical, oval, and curved
Optimum temperature for growth (°C)	25~27	25~27	25~27	25~27

+++; excellent; ++, good; +, no sporulation. Excellent, more than 100 spores; Good, more than 50 spores. PDA, potato dextrose agar; OMA, oat meal agar; CMA, corn meal agar.

30 min at room temperature, followed by centrifugation for 20 min at 4,500 ×g. The supernatant was then transferred into a fresh tube, and 0.6 vol of isopropanol was added to precipitate the DNA. The precipitated DNA was pelleted and washed twice with 70% ethanol, air dried, and dissolved in 1~2 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at 55°C. The quality of the DNA was checked on 0.8% agarose gel stained with ethidium bromide.

PCR amplification of internal transcribed spacer (ITS) regions and 18S rRNA. Universal primers were used for amplification of the ITS and 18S rRNA regions from the fungal strain using a Robocycler Gradient 96 (Stratagene, Cedar Creek, TX, USA). PCR reaction was set up in 15 µL volume consisting of 40 ng of fungal DNA (0.4 µL), 1.5 µL of 10× buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM MgCl₂, 0.1% [w/v] gelatin, 0.8% Triton X-100), 4 µL of (0.2 mM) dNTPs (Sigma, St. Louis, MO, USA), 1 µL of (1 µM) each primer set (for ITS [forward] ITS1-5'TCCGTAGGTGAACCTGCGG3'/[reverse] ITS2-5'GCTGCGTTCATCGATGC3', [forward] ITS3-5'GCATCGATGAAGAACGCAGC3'/[reverse] ITS4-5'TCCTCCGCTTATTGATATGC3' [18] and for rRNA [forward] NS1-5'GTAGTCATATGCTTGCTC3'/[reverse] NS4-5'CTTCCGTC AATTCCTTTAAG3' [19]), and 0.5 U/µL of Taq polymerase (Bangalore Gene, Bangalore, India). PCR was carried out under the following conditions: for ITS regions, initial denaturation at 95°C for 3 min, 36 cycles: 95°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec; final extension was at 72°C for 3 min; for 18S rRNA: initial denaturation at 95°C for 3 min, 36 cycles: 95°C for 30 sec, 52°C for 30 sec, 72°C for 1.30 min; final extension

was at 72°C for 10 min. The resulting PCR products were analyzed on 1% agarose gel containing ethidium bromide. PCR fragments were eluted from gel with the help of a gel elution kit according to the manufacturer's instructions (Bangalore Gene). The purified PCR products were cloned into pGEMT vector (Promega, Madison, WI, USA) and transformed into DH5α competent cells [20]. Plasmid containing the insert was isolated using the alkali lysis method [20]. The amplified DNA fragments were visualized on 1.2% agarose gel containing ethidium bromide.

DNA sequencing and sequence analysis. The cloned fragments were sequenced using Sanger's dideoxy method [21] using a sequencing kit (ABI Prism Big Dye Terminator Cycle seq, ABS; Applied Biosystems, Foster City, CA, USA). The most identical sequences of the strain AAT-TS-4₁ were identified from the NCBI database of Genbank using the BLAST algorithm [22]. The nucleotide sequences showing high sequence similarity were manually picked for further analysis. Multiple sequence alignment was performed with nucleotide sequences of other closely related species obtained from the NCBI-GenBank database using the ClustalW algorithm [23]. Phylogenetic analysis was carried out using PHYLIP ver. 3.62 suite programs [24]. Since the sequences showing maximum similarity in BLAST search were selected, DNAPARS program was used for further analysis. The bootstrap values for the parsimony tree were obtained by analysis of 100 replicates with input order jumbled 10 times using the SeqBoot program. A consensus tree was constructed from the output generated using the CONSENSUS program. DNAML, a program based on maximum-likelihood method, was also used for comparison. Both methods were used to analyze

all three sequences (two ITS region sequences and one 18S rRNA sequence) of the fungus AAT-TS-4₁. The data generated was converted into trees using DRAWGRAM.

Results

Endophytic fungi from *T. baccata*. Out of 40 endophytic fungal cultures screened for taxol production, one culture assigned as AAT-TS-4₁ (an endophyte isolated from the stem) was confirmed to produce taxol. None of the other fungal cultures produced considerable concentrations of taxol.

Cultural and morphological characters of the fungus.

The fungus AAT-TS-4₁ isolated and grown on PDA medium produced slow-growing colonies (Fig. 1A). Colonies on PDA were white, flocculose, circular, compact, reverse cream, margin smooth, hyphae branched septate, smooth, and hyaline [25]. Non-stromatic conidiophores produced from superficial hyphae were erect, branched to unbranched, septate, and hyaline. Phialides solitary or produced in a group of 2~5 were straight, smooth, verticillate, and hyaline. Conidia produced singly or in a small moist clusters apically were smooth, oval to cylindrical to curved, hyaline, single-celled, and variable in shape and size [26].

Based on cultural and morphological characteristics, the strain was identified as *Gliocladium* sp.

Effects of various nutrient media on growth and sporulation.

The fungus showed variation in growth rate when grown on various nutrient media. The results are presented in Table 1. Among the four different media tested, it was found that OMA media was the best for growth of the fungus, followed by CMA, PDA, and V-8 juice media. Excellent sporulation was recorded on PDA and OMA media. Further, good sporulation was observed in CMA medium and no sporulation was recorded in V-8 juice medium.

PCR amplification and sequence analysis.

The method employed for genomic DNA isolation resulted in high quality DNA. The two different ITS regions (0.2 and 0.3 kb) and 18S rRNA regions (1.1 kb) were amplified using universal primers. BLAST analysis showed the majority of the hits from a group of fungi belonging to Ascomycetes, which included *Gliocladium* sp., *Bionectria* sp., *Nectria* sp., and *Clonostachys* sp. All three nucleotide sequences (ITS 1 & 2, ITS 3 & 4, and NS1 & NS4) showed similarity in the range of 95~98%. The nucleotide sequence analysis determined that the 0.2 kb fragment was a partial

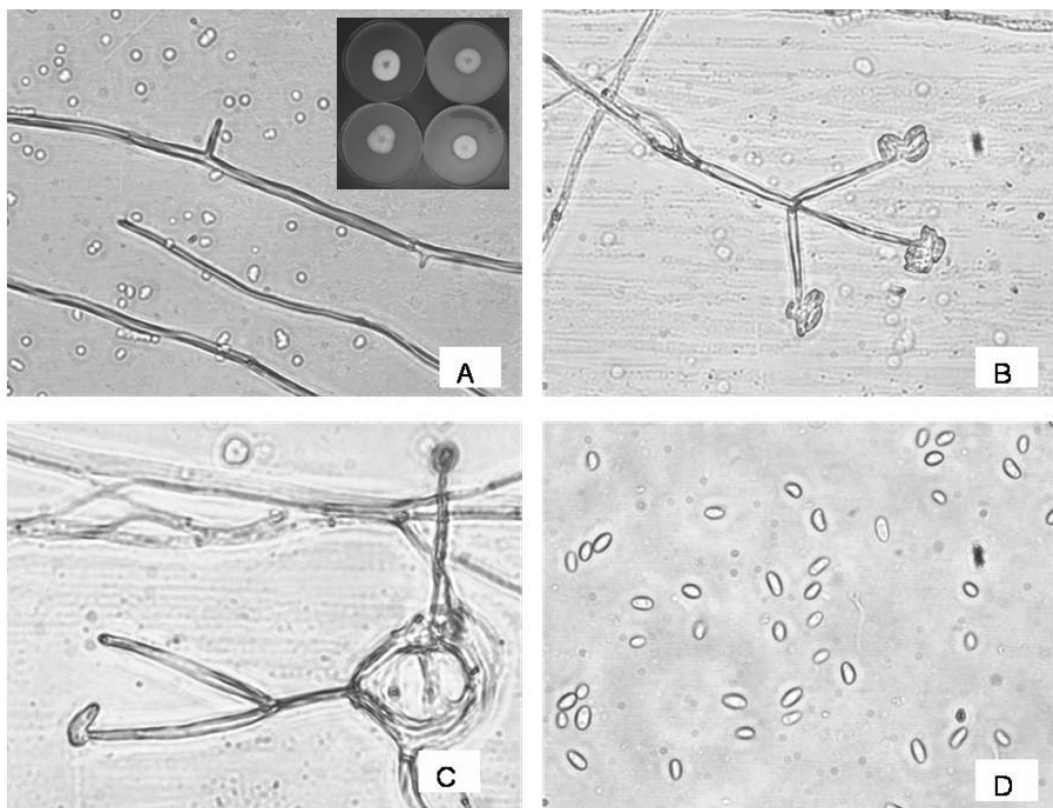


Fig. 1. Microscopic observations of morphology of fungus AAT-TS-4. A, Mycelium; B, Conidiophores bearing conidia; C, Arrangement of conidia on phialide tip; and D, Conidia. The inset in A showing the picture of *Gliocladium* sp. growing on potato dextrose agar, oat meal agar, corn meal agar, and V-8 juice (clockwise).

ITS region at the 5' end and partial 5.8S rRNA gene at the 3' end, the 0.3 kb fragment was a partial 5.8S rRNA gene at the 5' end and partial ITS region at the 3' end, and the 1.2 kb fragment was a 18S rRNA gene at the 5' end

and partial 28S gene at the 3' end. All of the sequences were aligned with ClustalW, and the relatedness between the sequences was determined. All sequences were submitted in NCBI gene bank and are retrievable with accession numbers EU528675, EU581866, and EU581865.

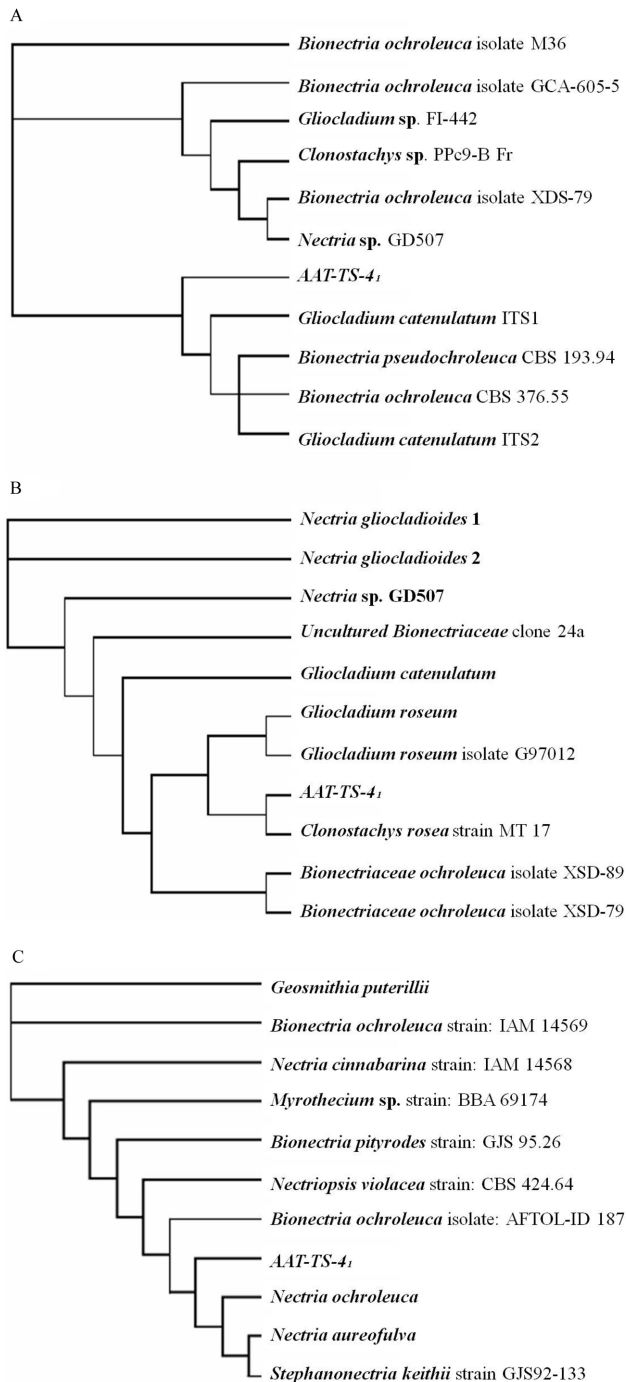


Fig. 2. Phylogenetic relationships of selected members with fungi AAT-TS-4₁. The trees were generated by parsimony algorithm (DNAPARS). A, B, The rooted tree showing relatedness of the AAT-TS-4₁ ITS region sequences with the genera *Gliocladium* and *Clonostachys*; C, Phylogenetic tree showing relatedness of 18S rRNA gene with genera *Nectria* and *Bionectria*.

Phylogenetic analysis. Fig. 2A and 2B shows the results of the phylogenetic analysis of the ITS 1 & 2 and ITS 3 & 4 sequences of the ITS region. The overall grouping of the fungi was the same for both methods (DNAPARS and DNAML); however, the precise ordering of the fungi was different. Sequence comparison with other closely related species confirmed that most of the sequences from the database showed higher than 95~98% sequence identity, but only *Gliocladium* sp. and *Clonostachys* sp. were grouped together with the ITS region sequence of AAT-TS-4₁. To be more specific, *Gliocladium* sp. showed an outstanding relatedness with AAT-TS-4₁ and shared a common clade. Further, all *Nectria* sp. (teleomorph) sequences were grouped together with *Gliocladium* and AAT-TS-4₁ sequences (Fig. 2B). In the case of 18S rRNA sequence, AAT-TS-4₁ was always grouped with *Nectria* sp. in both methods of analysis (Fig. 2C), which was a teleomorph of *Gliocladium* sp. Even though fungi belonging to genera *Myrothecium*, *Geosmithia*, and *Stephanonectria* showed higher than 95% sequence similarity, none of them showed relatedness with AAT-TS-4₁ in phylogenetic analysis.

Production of taxol. Taxol concentrations increased with an increase in culture incubation time. Low concentration of taxol was detected in the culture after as early as 2 days of incubation. Approximately 118, 338, 434, 486, and 1,076 ng/200 mL of taxol were estimated by ELISA in culture after 2, 5, 10, 15, and 21 days of incubation,

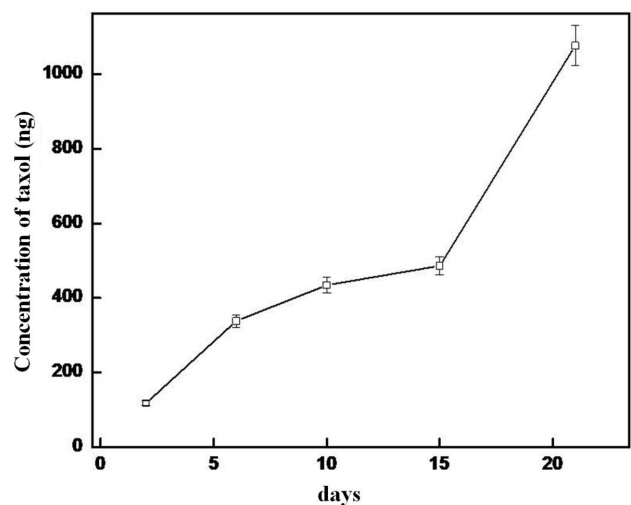


Fig. 3. Production of taxol by the fungal strain *Gliocladium* sp. at different time intervals. Data represents mean values from five separate experiments \pm SD.

respectively (Fig. 3). Taxol in mycelium and culture broth was estimated to be 481 and 1,670 ng/200 mL, respectively. Exact concentrations of the taxol in the extracts were not determined as the solubility of the chloroform extracts was very poor in methanol.

Discussion

In the present study, slow growing and unusual endophytic fungal cultures were isolated from different tissues (stem, bark, and needles) of *T. baccata*. The endophyte population explored from the *Taxus* sp. represents different genera of fungi. Forty endophytic fungal strains were screened for taxol production using immunoassay. Among the fungal cultures, one strain designated as AAT-TS-4₁ was detected as taxol-producing. Taxol production increased exponentially with an increase in growth of the fungal culture (21 days), indicating that taxol was produced in maximum quantities at later log phase or early stationary phase of fungal growth. However, as taxol was observed in culture after as early as 2 days of incubation, these concentrations were much higher in comparison with other fungal strains reported to produce ng quantities of taxol after 21 days of culture [15]. Taxol production was estimated to be high in culture broth as compared to fungal mycelium, indicating that the metabolites were secreted into the medium. The culture on solid media took more than 1 wk to reach optimum growth, which suggests its identity as an endophyte [27]. Microscopic analyses showed that the mycelia were hyaline and septate, bearing verticillium-like conidiophores, a characteristic feature of *Gliocladium* sp. However, we did not observe any secondary penicilliate conidiophores. Phialides were solitary or produced in a group of 2~5, straight, smooth, and verticillium-like. Conidia were produced singly or clustered apically and were smooth, oval to cylindrical to curved, hyaline, single-celled, and variable in shape and size, typical of *Gliocladium* sp. All of the mentioned characteristics were identical to those described for the fungus *Gliocladium* sp. [25, 28]; hence, fungus AAT-TS-4₁ could be referred to as *Gliocladium* sp. As some of the morphological characteristics such as conidiophore structure and shape of conidia resembled or were very similar to another fungus belonging to genus *Verticillium* sp., we found it difficult to identify the fungus based on morphological characteristics alone. Thus, we analyzed the ITS and 18S rRNA regions to confirm the identity of the fungus.

In sequence analyses using BLAST and ClustalW, both ITS sequences (0.2 kb and 0.3 kb sequences) and 18S rRNA sequence (1.1 kb) show highest similarity with genera - *Gliocladium*, *Bionectria*, and *Clonostachys*. Phylogenetic studies indicated that the nearest relative of the fungus AAT-TS-4₁ was either from genus *Gliocladium* or *Clonostachys*. Considering morphological characteristics,

we identified the fungus as *Gliocladium* sp., as the fungus did not show any asci formation, which is very often seen in *Bionectria*, and also did not produce conidia in columns as reported in *Clonostachys* [29]. 18S rRNA phylogenetic analysis showed that the fungus was closely related to genus *Nectria* sp., a teleomorph (perfect state) of *Gliocladium* sp. The sequence analysis and phylogenetic analysis supported the identification. However, in recent reports, many species of genus *Gliocladium* have been classified under *Clonostachys* and *Bionectria* based on morphological and molecular similarities [26]. To the best of our knowledge, this is the first report of the isolation of taxol from *Gliocladium* sp.

Stierle *et al.* [15] and others have isolated taxanes from different endophytic fungal species (*Taxomyces andreanae*, *Pestalotiopsis*, *Pestalotia*, *Fusarium*, *Alternaria*, and others) obtained from *Taxus* sp., which are common to Europe, Asia, and North America. The isolation of taxol from the culture of *Gliocladium* sp. is the first demonstration of its occurrence in fungi isolated from Indian Yew tree *T. baccata*. The other interesting aspect of our study is the occurrence of *Gliocladium* sp. as an endophyte, since some *Gliocladium* sp. have been isolated as mycoparasites producing novel secondary metabolites. The discovery of such a mycoparasitic fungus being associated as an endophyte might help us understand the mode of transformation of genetic material between the endophyte and the host, as many of these endophytes mimic the chemical diversity of the host. In conclusion, we isolated an endophytic fungus *Gliocladium* sp. producing taxol extra/intracellularly.

Acknowledgements

D. Sreekanth, G. K. Sushim, and A. Syed thank the Council of Scientific and Industrial Research (CSIR), Government of India for a Research Fellowship. Financial support from the Department of Biotechnology (DBT), New Delhi, India is gratefully acknowledged.

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