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

Sanjog Garyali, Anil Kumar, and M. Sudhakara Reddy*

Department of Biotechnology, Thapar University, Patiala 147004, Punjab, India

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 irreplacable 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-deacetylbaccatin III-10-O-acetyl transferase (DBAT) and C-13 phenylpropanoic 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 diterpene 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'-deoxytaxol, that is, it catalyzes the attachment of the biologically important taxol side chain precursor [26].

**Materials and Methods**

**Isolation of Endophytic Fungi**

Bark samples (1 × 3 cm) were harvested from the stem of relatively young *T. bacatta* subsp. *wallichiana* from Bhadrewha (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-pestle 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’-GGGAGG GTGCTCTGTTTG-3’ and *dbat*-R 5’-GTACCCTGAAACCACCA GG-3’) and the *BAPT* gene (*bapt*-F 5’-CCTCTCCTGCCATTTGAC AA-3’ and *bapt*-R 5’-TCGCC ATCTGGC CCACTTGT3’) were as described by Li et al. [11]. Thermal-cycles 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, 55°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 Fungi**

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