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Induction of Taxol biosynthesis by *Aspergillus terreus*, endophyte of *Podocarpus gracilior* Pilger, upon intimate interaction with the plant endogenous microbes

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ABSTRACT

Twenty-four endophytic fungal isolates recovered from *Podocarpus gracilior* were screened for Taxol biosynthetic potency. Among these endophytes, three isolates of *Aspergillus terreus* EFB108, EFB59, EFB14 recovered from *P. gracilior* cork, leaves and twigs, respectively, were reported as potent Taxol producers. The chemical identity of *A. terreus* extracted Taxol was verified from the HPLC, NMR and FTIR analyses, with strong cytotoxic activity for HEPG2 and MCF7 cell lines (IC_{50} 4.5–6.5 nM). Upon nutritional of *A. terreus* EFB108 (MF377552), the maximum yield of Taxol (265 µg/L) was obtained by growing the fungus on MID medium of initial pH 8.0, with 0.6% asparagine and 2.4% xylose, incubated for 21 days. Taxol yield of *A. terreus* was increased by ~2.4 folds (432 µg/L) upon addition of surface sterilized *P. gracilior* leaves. The yield of Taxol by *A. terreus* was not affected by the dichloromethane plant extract negating the presence of direct signals inducing Taxol biosynthesis. So, the intimate physical interaction of endogenous endophytes of *P. gracilior* with the fungus, could be essential for triggering the biosynthesis of Taxol by *A. terreus*. This is the first report describing the feasibility of *A. terreus*, endophytes of *P. gracilior*, for Taxol production that could be a good industrial platform.

1. Introduction

Taxol is a highly oxygenated diterpenoid (C20) that firstly isolated from Pacific Yew trees, *Taxus brevifolia* [1] and approved by US FDA in 1994 as blockbuster anticancer drug [2] for metastatic ovarian carcinoma, breast and lung cancer. The activity of Taxol elaborates from its unique specificity for binding with tubulin β-subunits heterodimer, promoting tubulin polymerization, disrupting mitotic division of target cells [3]. Taxol is naturally produced from the bark of *T. brevifolia*, however, its tiny yield and vulnerability of this plant to unpredicted fluctuation with the ecological conditions were the major challenges for this source [4,5]. Semisynthetic process based on 10-decaetylbaccatin III (DAB) intermediates extracted from the needles of European Yew (*Taxus baccata*) is the current technology for Taxol production, however, the strong heterogeneity of DAB yield with environmental conditions of *T. baccata*, requiring wide cultivation land area and breading time are the current challenges [6]. Alternatively, fungal endophytes from *Taxus* spp. with strong potency for Taxol production opened a new avenue to scale-up the Taxol yield, due to their fast growing, cost effective process, independent on climatic changes and possibility for growing on bulk fermenters, resistance to shearing and feasibility for genetic manipulation [7]. However, the anticipation of endophytic fungi for industrial scale production of Taxol has been challenged by their lower reproducible yield and drastic loss of Taxol productivity with repeated subculturing [8–10].Thus, searching for novel fungal endophytes from different medicinal plant hosts with promising, sustainable Taxol yield is the ultimate objective.

Plants of Podocarpaceae, with an ethnopharmacological relevance, were traditionally assigned to their biological activity against various bacterial and fungal pathogens [11]. The bark and stems extracts of *Podocarpus* spp. were traditionally used as remedies for stomachache, cattle disease, gonorrhea, deworming and cancer treatment [12], especially *Podocarpus gracilior*, *P. neriifolius, P. macrophyllus*, *P. elongatus* and *P. falcatus* were the most recognized species for these purposes [11,13,14]. Endophytic fungi of *P. falcatus* were isolated displaying a remarkable activity against various phytopathogens [15]. Taxol has been identified from *P. gracilior* (African Fern Pine) [12], and this was the first report to identify Taxol from plants outside the family

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Taxaceae, revealing the closely taxonomic relations of Podocarpaceae and Taxaceae [16]. However, the lower concentration of Taxol in *P. gracilior* leaves and stems (> 0.54 mg/kg dry weight tissues) preclude its further uses for commercial production [14,11]. From literature, there is no reports describing the endophytic fungi of *Podocarpus* species. Thus, searching for a potential fungal endophyte from *Podocarpus* species producing Taxol with appreciable yield might be the alternative prospective technology to conquer the lower accessibility of this drug. Therefore, the objective of this study was to isolate and characterize Taxol-producing endophytic fungi from *P. gracilior*, studying the nutritional requirements provoking the yield of Taxol by the selected fungal isolate.

2. Materials and methods

2.1. Isolation of the endophytic fungi inhabiting Podocarpus gracilior

Different parts of *P. gracilior* such as leaves, barks and twigs were collected in October-December/2016 (El-Zohria Garden, Cairo, Egypt) and used as source for isolation of endophytic fungi. The plant parts were collected and surface sterilized with 70% ethanol for 2 min, followed by 2.5% sodium hypochlorite for 4 min, then rinsing two times with sterile distilled water [17]. The effectiveness of surface sterilization of the plant parts was assessed, by centrifugation of the rinsing water, then 500 µl of sterile water was added to the precipitate and plated into potato dextrose agar [17]. The parts were segmented with sterile sharp blade, placed on the surface of Potato Dextrose Agar (PDA), Czapek's-Dox and malt extract agar [18], incubated for 15 days at 30 °C. Ampicillin (1 µg/ml) was routinely added to the media prior pouring into the plates. The developed hyphal tips of the fungal colonies were purified by subculturing on the corresponding media with daily inspection till 15 days of incubation. The purified endophytic fungal isolates were stored as slope and plate cultures at 4 °C. Control plate media free of the plant parts were used to check the sterility of working area. Positive control of plant parts without sterilization were used to check the epiphytic fungal flora.

2.2. Morphological and molecular identification of the recovered endophytic fungi

The purified fungal isolates were grown on Czapek's-Dox and PDA media for 15 days at 30 °C. The developed fungal colonies were examined daily based on the microscopical features as colony diameter, extracellular exudates, pigmentation, mycelium color, conidial heads, fruiting bodies and sporulation [19,20]. The fungal isolates were identified to the genus and species levels based on the morphological features [21,18,22,23].

The recovered fungal isolates were molecularly identified based on the entire sequence of internal transcribed spacer (ITS) flanking the 5.8S region (ITS1-5.8S-ITS2 rDNA) [24]. Fungal genomic DNA (gDNA) was extracted [25], used as template for PCR amplification, with the primer set ITS4 5′-GGAAGTAAAAGTCGTAACAAGG-3′ and ITS5 5′-TCCTCCGCTTATTGA TATGC-3′ [24]. The PCR reaction contains 10 µl of 2× PCR master mixture (*i*-Taq™, Cat. No. 25027, INTRON Biotech.), 2 µl of fungal gDNA, 1 µl of forward and reverse primers (10 pmol/µl), completed to 20 µl with sterile distilled water. The PCR was programmed to initial denaturation at 94 °C for 2 min followed by denaturation at 94 °C for 30 s, annealing at 55 °C for 10 s, extension at 72 °C for 30 s for 38 cycles, with final extension at 72 °C for 2 min. The PCR amplicon was analyzed by 1.5% agarose gel in 1× TBE buffer (Ambion Cat# AM9864) comparing to the DNA ladder (1 kb Nex-gene Ladder, Puregene, Cat.# PG010-55DI). Negative control PCR reactions (without fungal gDNA) was used. The amplicons were visualized by gel documentation system (Vilber Lourmat, France), purified and sequenced by Applied Biosystems Sequencer, HiSQV Bases, Version 6.0 using the same primer sets. The obtained ITS sequences were BLAST searched with non-redundant sequences on the NCBI database. The quality of retrieved sequences was visually inspected from the sequence chromatograms. For the multiple sequences alignments, FASTA sequences were imported into MEGA 6.0 software and aligned with Clustal W muscle algorithm [26]. The phylogenetic tree of the target sequences was constructed with neighbor-joining method of MEGA 6.0 with 1000 bootstrap replication [27].

2.3. Cultivation of endophytic fungi, taxol extraction and quantification

The recovered endophytic fungi from *P. gracilior* were screened for Taxol production on Potato dextrose broth (PDB) (BD Difco, Cat# DF0549-17-9) [9], Czapek's Dox medium [18] and M1D medium supplemented with 0.1% soytone [24,25,7]. The M1D medium contains (g/l): sucrose 30 g, ammonium tartrate 5 g, yeast extract 0.5 g, soytone 1.0 g, $Ca(NO_3)_2$ 0.2 g, KNO_3 0.08 g, KCl 0.06 g, $MgSO_4$ 0.36 g, NaH_2PO_4. H_2O 0.02 g, $FeCl_3$ 0.002 g, $MnSO_4$ 0.005, $ZnSO_4$. $7H_2O$ 0.003 g, H_3BO_3 0.014 g and KI 0.0001 g, dissolved in distilled water. Each fungal isolate (2 agar plugs of 5 mm) of 6 days old grown on PDA, were inoculated into 50 ml medium/250 ml Erlenmeyer flask, incubated at 30 °C under static condition for 20 days. After incubation, the cultures were filtered through sterile cheesecloth to remove the fungal mycelia, the filtrates were amended with 0.03% sodium bicarbonate to precipitate fatty acid and Taxol has been extracted with double volume of dichloromethane (DCM). The organic phase was collected, solvent was evaporated to dryness at 35 °C and the residues were re-dissolved in 3 ml of methanol.

Taxol was identified by TLC analysis [29] using Merck 1 mm (20 × 20 cm) pre-coated silica gel plates (TLC Silica gel 60 F_{254}, Merck KGaA, Darmstadt, Germany). The TLC plates were developed with the solvent system methylene chloride/methanol/dimethyl formamide (90:9:1, v/v/v). After running, Taxol was detected by UV illumination at 254 nm (Min-UVIS, DESAGA, Heidelberg, Germany), in addition, the plates were sprayed with 1% vanillin in sulfuric acid (w/v), gentle heating [30], a bluish spot fading to dark gray were developed after 24 h, comparing to authentic Taxol (Sigma-Aldrich, Cat. # T7402).

The putative spots of silica containing Taxol were scraped-off from the plate and dissolved in methylene chloride, vortex thoroughly for 3 min. The purity and concentration of extracted Taxol were analyzed by HPLC (Agilent Technology, G1315D) of C18 reverse phase column (Eclipse Plus C18 4.6 × 150 mm, 3.5 µm, Cat.# 959963-902) with isocratic mobile phase of methanol/acetonitrile/water (25:35:40, v/v/v) at flow rate 1.0 ml/min for 20 min [31]. Taxol fractions were scanned from 200 to 500 nm by photodiode array detector (DAD), their chemical identity and concentrations were confirmed from the retention time and absorption peak area at 227 nm.

2.4. Spectroscopic analysis

The UV-absorption of Taxol samples was measured at λ200 to λ300 nm by Spectrophotometer (RIGOL, Ultra-3000 Series), displaying a characteristic peak at 227 nm, comparing to authentic Taxol. Blank media was used as negative control and as baseline for spectrophotometer analysis.

The Infra-Red (FT-IR) spectra of purified Taxol sample were analyzed using a JASCO, FTIR 6100 Spectrophotometer (National Research Center, Egypt). The purified Taxol sample was grinded with KBr pellets pressed into discs under vacuum, comparing to authentic one. The IR spectra was recorded in the region between 4500 and 500 cm^{-1} [1,28].

The chemical structure of extracted Taxol was confirmed from the ^{1}H and ^{13}C NMR spectra (JEOL, ECA-500II, 500 MHz NMR at Faculty of Science, Mansoura university, Egypt) comparing to authentic Taxol. The samples were dissolved in CDCl3, chemical shifts are given in ppm (δ-scale) and the coupling constants are expressed in hertz (Hz).