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研究报告

# Taxol Determination from *Pestalotiopsis pauciseta*, a Fungal Endophyte of a Medicinal Plant

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**Abstract:** Taxol is the most effective antitumor agent developed in the past three decades. It has been used for effective treatment of a variety of cancers. A taxol-producing endophytic fungus *Pestalotiopsis pauciseta* (strain CHP-11) was isolated from the leaves of *Cardiospermum helicacabum* and screened for taxol production. The fungus was identified based on the morphology of the fungal culture and the characteristics of the spores and screened for taxol production. The amount of taxol produced by this endophytic fungus was quantified by HPLC and it produced 113.3 μg/L, thus the fungus can serve as a potential material for fungus engineering to improve taxol production. This fungal taxol also had strong anticancer activity against some cancer cells viz., BT 220, H116, Int 407, HL 251 and HLK 210 tested by Apoptotic assay and it is indicated that with the increase of taxol concentration from 0.005–0.05 μmol/L, taxol induced increased cell death through apoptosis.

**Keywords**: endophytic fungus, medicinal plant, taxol production, liquid medium

The study of endophyte distribution, biodiversity and their biochemical characteristics are of immense importance in plant biology to understand and to improve plant fitness[1]. The endophytic fungi are of biotechnological importance as new pharmaceutical compounds, secondary metabolites, agents of biological control and other useful characteristics could be found by further exploration of endophytes. There is a general call for new antibiotics, chemotherapeutic agents and agrochemicals that are highly effective, possess low toxicity, and will have a minor environmental impact. Endophytic fungi are relatively unexplored producers of metabolites useful to pharmaceutical and agricultural industries[2]. A very conservative consumption of two or three unique endophytic species per plant translates to some 750 000 endophytic fungi potentially as sources of novel secondary metabolites. A background understanding that involves some specific examples and rationale of the presence of endophytic microorganisms in higher plants will aid in the development of a drug discovery program involving these organisms. Taxol, an important and expensive anticancer drug, was originally isolated from *Taxus brevifolia*[3]. Taxol is produced by all yew species but it is found in extremely low amounts in the needles, bark and roots[4,5].

Presently, all taxol in the world's market has originated from *Taxus* spp. Although complete chemical synthesis of taxol has been achieved, the process is too expensive for commercialization. If the fungus can be manipulated to increase the production of taxol by some technique, it can provide an inexhaustible source of taxol and the supply problem of this drug can be solved forever. Since the first taxol-producing fungus *Taxomyces andreanae* was isolated in 1993[6,7], there have been a few reports on the isolation of taxol-producing endophytic fungi, demonstrating that organisms other than *Taxus* sp. could produce taxol[8–10]. Virtually very few reports are available on screening taxol-producing endophytic fungi from tropical medicinal plant species. Therefore, this study provides first report on taxol production by fungal endophyte of medicinal plant from southern India. Keeping this in view, the aim of the present investigation was to identify the potential taxol-producing endophytic fungus, *Pestalotiopsis pauciseta* isolated from the leaves of a medicinal plant, *Cardiospermum helicacabum* L. (*Sapindaceae*). The leaves are rubefacient and good for arthritis, ophthalmodynia and useful in strangury, fever, amenorrhoea, lumbago and neuropathy.

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# 1 Materials and methods

## 1.1 Isolation and identification of endophyte

The endophytic fungal culture used in this study was isolated from the leaves of medicinal plants in Chennai city, India. The leaf samples were surface sterilized by Suryanarayanan *et al*[11]. The surface sterilized leaf segments were evenly spaced in petri dishes (9 cm diameter) containing potato dextrose agar (PDA) medium (amended with chloramphenicol 150 mg/L). The petri dishes were sealed using Parafilm™ and incubated at 26±1°C in a light chamber with 12 hours of light followed by 12 hours of dark cycles. The petri dishes were monitored every day to check the growth of endophytic fungal colonies from the leaf segments. The hyphal tips, which grew out from leaf segments were isolated and identified using standard monographs. The identified fungal cultures were deposited at the Madras University Botany Laboratory (MUBL), CAS in Botany, University of Madras, Chennai-600 025. The immediate concern is to find one or more fungi that produce more taxol. An endophytic fungus *P. pauciseta* strain CHP-11 (MUBL No. 682), was screened for taxol production. Photomicrographs of conidia were taken with the help of Carl Zeiss Axiostar plus-Photomicroscope (phase contrast) with Nikon FM 10 Camera and Nikon HFX Labophot (bright field) with Nicon FX-35A by using Konica films.

# 2 Extraction of fungal taxol

The endophytic fungus was grown in 2 L Erlenmeyer flasks containing 500 mL of MID medium supplemented with soytone[12] and incubated for 21 days. After 3 weeks of still culture at 26°C, the culture fluid was passed through four layers of cheese cloth to remove solids and extracted with organic solvent. The extraction and isolation procedure followed was that of Strobel *et al*[13]. After methylene chloride extraction, the organic phase was collected and the solvent was then removed by evaporation under reduced pressure at 35°C using rotary vacuum evaporator. The dry solid residue was re-dissolved in methanol for the subsequent separation and extracts were analyzed by chromatographic separation and spectroscopic analyses. The standard taxol (Paclitaxel) was purchased from SIGMA.

## 2.1 Chromatographic analyses

All comparative Thin Layer Chromatographic analyses were carried out on Merck 0.25 mm silica gel plates, developed in the following solvents: a. chloroform/methanol (7:1 *V*/*V*); b. chloroform/ acetonitrile (7:3 *V*/*V*); c. ethylacetate/2-propanol (95:5 *V*/*V*); d. methylene chloride / tetrahydrofuran (6:2 *V*/*V*); e. methylene chloride/methanol/dimethylformamide (90:9:1*V*/*V*/*V*). The presence of taxol was detected with 1% *W*/*V* vanillin/sulphuric acid reagent after gentle heating[14]. To further confirm the presence of taxol, the fungal sample was analyzed by HPLC (Shimatzu 9A model) using a reverse phase $C_{18}$ column with a UV detector. The mobile phase was methanol/acetonitrile/water (25:35:40, by vol.) at 1.0 mL/min. The sample and the mobile phase were filtered through 0.2 μm PVDF filter before entering the column. Twenty microlitres of the sample was injected each time and detected at 232 nm. Taxol was quantified by comparing the peak area of the samples with that of the standard taxol.

## 2.2 Spectroscopic analysis

The purified sample of taxol was analyzed by UV absorption, dissolved in 100% methanol at 273 nm in a Beckman DU-40 Spectrophotometer and compared with standard taxol. The FAB mass spectra were recorded on a JEOL SX 102/DA-6000 Mass Spectrometer/ Data System using Argon/Xenon (6 kV, 10 mA) as the FAB gas. The accelerating voltage was 10 kV and the spectra were recorded at room temperature.

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# 3 Results and discussion

In addition to studying the distribution and ecology of fungal endophytes from medicinal plants, special attention should be given to screening them for potent metabolites. Endophytic fungi are increasingly recognized as sources of novel bioactive compounds and secondary metabolites for biological control[15]. The purpose of this present study is to isolate and identify the taxol-producing endophytic fungi from medicinal plants, so that the fungus can serve as a potential material for fungus engineering to improve the production of taxol. Based on the morphology of the mycelial colony as well as the characteristics of the conidia, the endophytic fungus was identified as *P. pauciseta*.

Conidiomata are acervular, amphigenous, black, scattered, sometimes gregarious, globose-lenticular, hemispherical, 80–200 μm diam. Conidia are 5-celled, not constricted at septa, erect or slightly curved, (19–24) μm × (6.5–7.5) μm; intermediate cells are coloured, 14–16 μm long, the upper two umber, lowest olivaceous, the walls are darker, broadest at septa dividing two upper coloured cells; exterior hyaline cells are conspicuous, apical cell is broad-conic, turbinate, bearing 3 setulae, 21–31 μm long; filiform, widely divergent, basal hyaline cells broad-conic,

obtuse or attenuated; pedicels up to 5 μm long (Fig. 1). This fungal culture was screened for taxol production and the extract of the fungus grown in liquid medium was examined for the presence of taxol by chromatographic and spectroscopic analysis. The compound has chromatographic properties identical to standard taxol in solvent systems A-E, and gives color reaction with the spray reagent and it appeared as a bluish spot fading to dark gray after 24 hours. They had $R_f$ values identical to that of standard taxol. Therefore, it was evident that this fungus showed positive results for taxol production. In HPLC analysis, the fungal extract isolated from *P. pauciseta* gave a peak with similar retention time as authentic taxol (Fig. 2). The UV spectral analysis of the fungal taxol is given in Fig. 3 and the spectrum was superimposed on that of authentic taxol at 273 nm. It was evident from high resolution mass spectrometry that the structure of taxol is more complex with empirical formula $C_{47}H_{51}NO_{14}$, corresponding to a molecular weight of 853.9. Characteristically, standard taxol yielded $MH^+$ at *m/z* 854. By comparison, fungal taxol also yielded a peak $MH^+$ at *m/z* 854 with characteristic fragment peaks at 569, 551, 509, 464, 286 and 268. Major fragment ions observed in the mass spectrum of taxol can be placed into three categories which represents major portions of the molecule[16]. The peaks corresponding to taxol, exhibited mass-to-charge (*m/z*) ratios corresponding to the molecular ions $(M+H)^+$ of standard taxol (854) confirming the presence of taxol in the fungal extracts. As reported in detail by Wani *et al*, the esterified position was found to be the allylic $C_{13}$ hydroxyl moiety[3]. The amount of taxol produced by this fungus in liquid culture was 113.3 μg/L. Strobel *et al* isolated *Pestalotiopsis microspora* from the inner bark of *Taxus wallachiana*, which was shown to produce taxol in mycelial culture and the total amount of taxol produced per litre was about 60–70 μg[13]. Commonly, taxol represents 0.01%–0.02% of the weight of dry bark, and the taxol content of 1 litre of *P. microspora* culture is about 0.001% of the total dry weight of the culture contents.

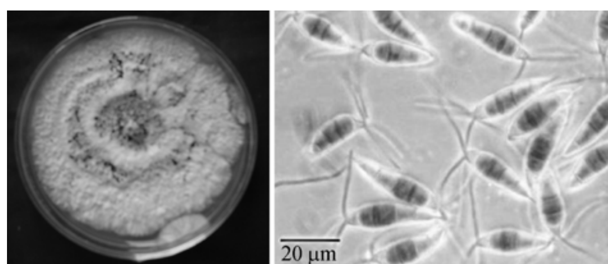


**Fig. 1 Growth of the fungus *Pestalotiopsis pauciseta* on petriplate and the conidia of the fungus**

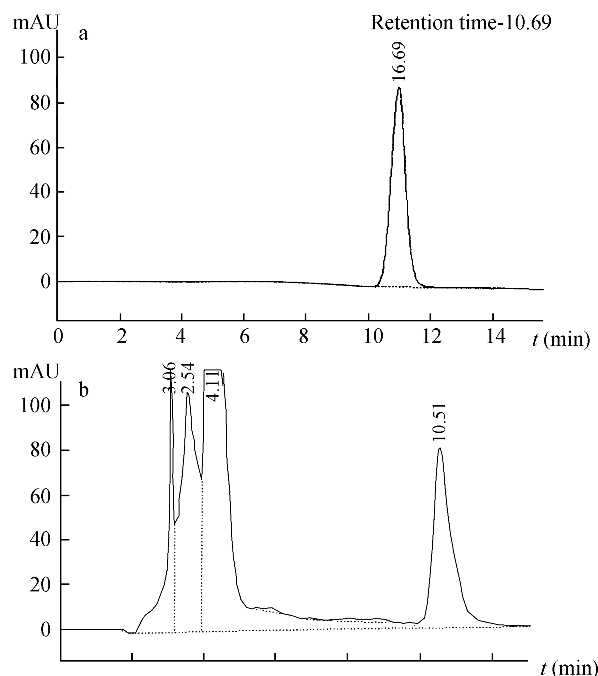


**Fig. 2 High performance liquid chromatogram with UV detection of authentic taxol (a) and fungal taxol extracted from *P. pauciseta* (b)**

the mobile phase was methanol/ acetonitrile/ water (25:35:40, *V/V*); Retention time of authentic taxol: 10.69 min; retention time of fungal taxol: 10.51

Additional persuasive spectroscopic evidence for the identity of taxol was confirmed by IR spectroscopy. The appearance of bands in IR spectra convincingly illustrates the identical feature of the extracted samples with authentic taxol. The IR spectral data of fungal taxol from *P. pauciseta* showed a broad peak in the region 3386.8 $cm^{-1}$ was ascribed to hydroxyl (-OH) and amide (-NH) groups stretch. The esters and ketone (C=O) groups stretch was observed in the region 1731.9 $cm^{-1}$. The aromatic ring (C=C) stretching frequency was observed in the region 1658.7 $cm^{-1}$. A peak observed in the region 1076.2 $cm^{-1}$ is due to the presence of aromatic C, H bends. The IR spectrum of *P. pauciseta* was superimposed on that of authentic taxol (Fig. 4).

Further convincing spectroscopic evidence for the identity of taxol was confirmed by FAB mass and $^1H$ NMR spectroscopy. The FAB mass spectrum of *P. pauciseta* was identical to that of authentic taxol (Fig. 5). Characteristically, authentic taxol yielded $MH^+$ at *m/z* 854. By comparison, fungal taxol also yielded a peak $MH^+$ at *m/z* 854 with characteristic fragment peaks at 569, 551, 509, 464, 286 and 268. $^1H$ NMR spectra were recorded at 23°C in $CDCl_3$ using a JEOL GSX 500 spectrometer (operating at 499.65 MHz). Almost all of the signals are well resolved and are distributed in the region from 1.0 to 8.5 ppm. The

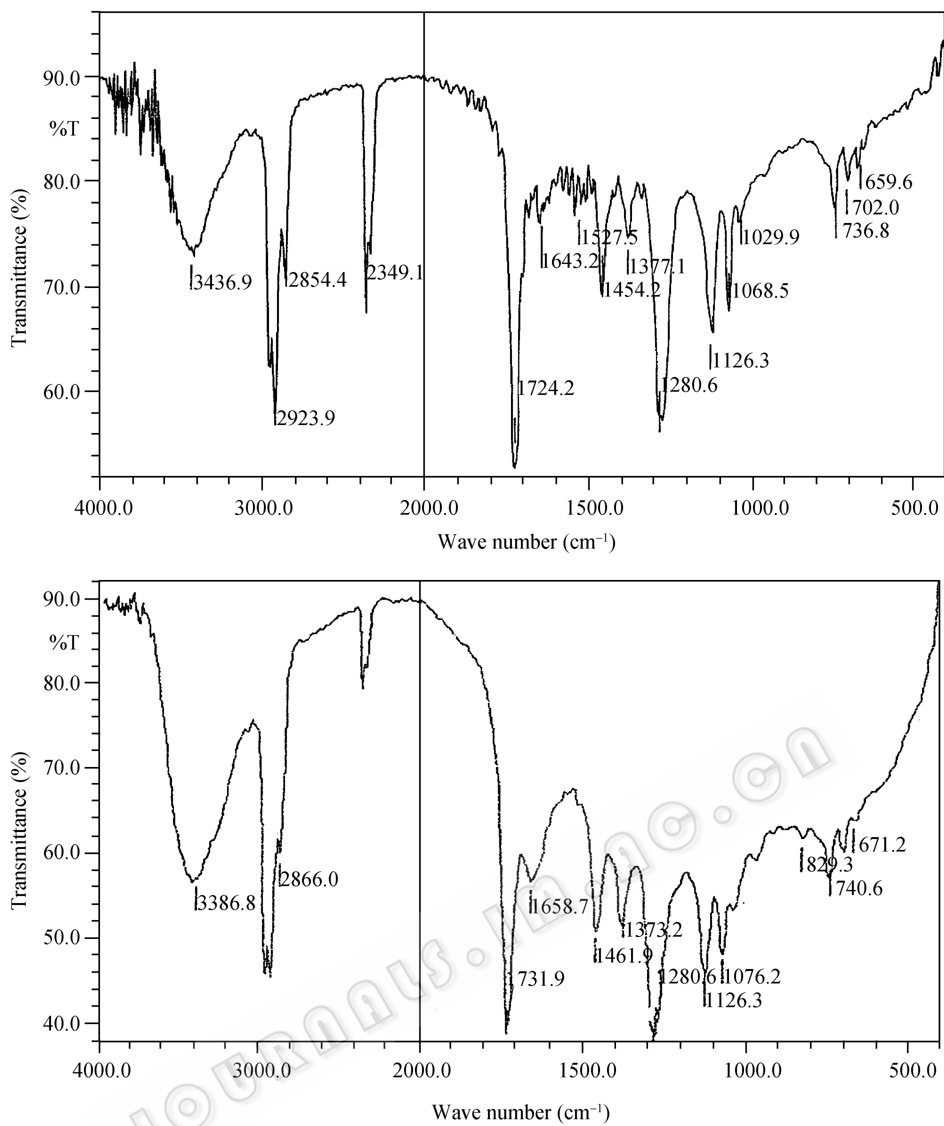


**Fig. 3 Ultra Violet absorption spectra of authentic taxol (a) and fungal taxol extracted from *P. pauciseta* (b)**

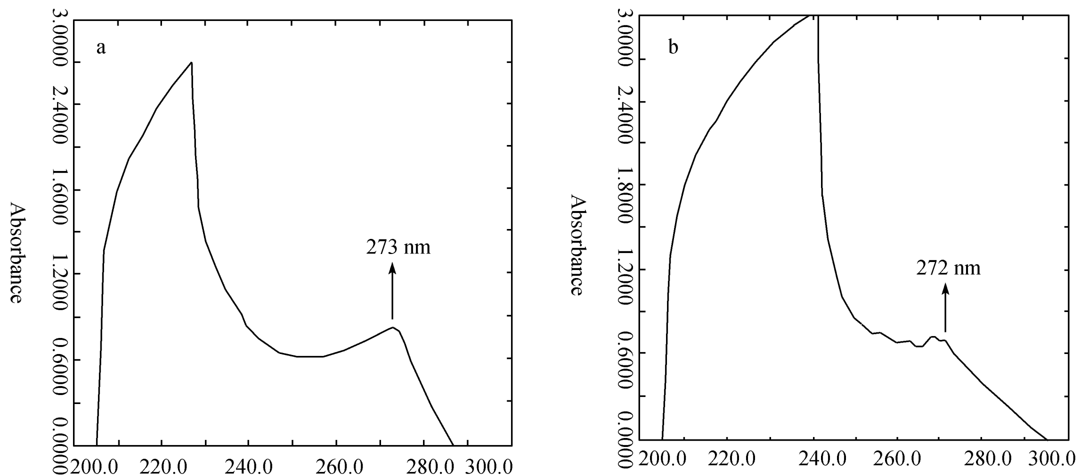


**Fig. 4 IR with UV detection of authentic taxol (a) and fungal taxol extracted from *P. pauciseta* (b)**

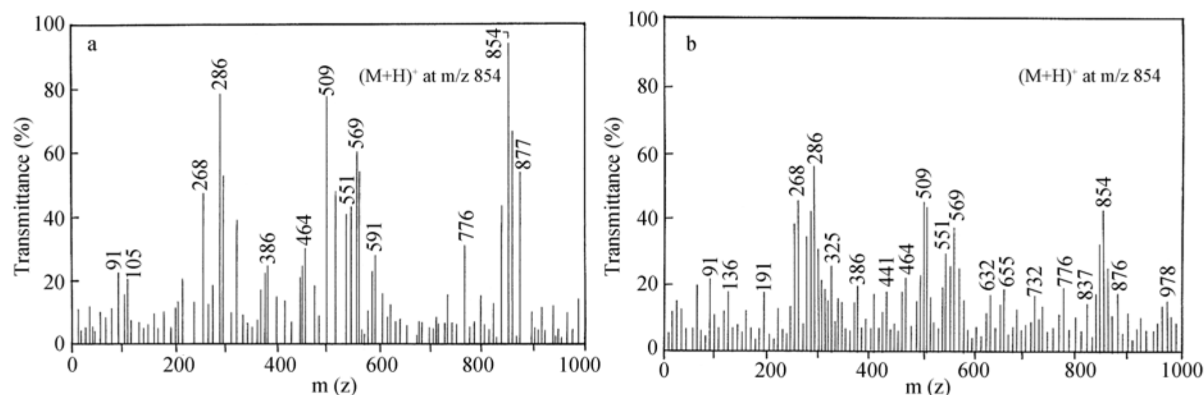


**Fig. 5 Fast Atom Bombardment Mass spectra of authentic taxol (a) and fungal taxol from *P.pauciseta* (b). The accelerating voltage was 10KV and recorded at room temperature**

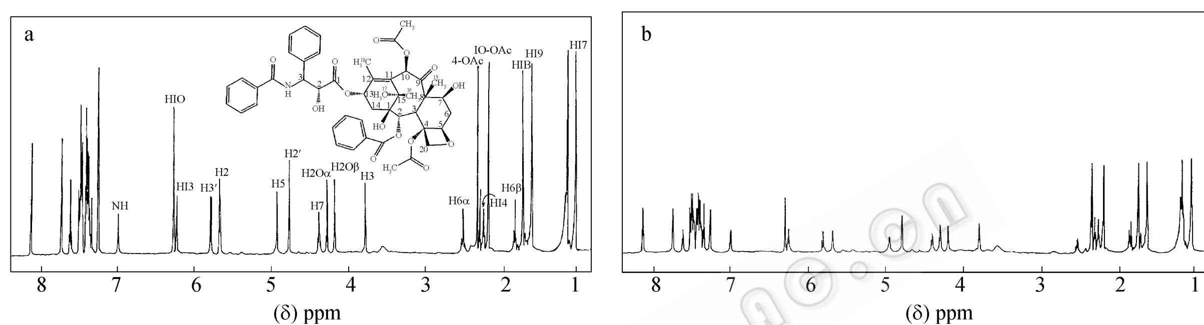


**Fig. 6 [1]H Nuclear Magnetic Resonance spectra of authentic taxol (a) and fungal taxol extracted from *P. pauciseta* (b). The structure of the taxol is shown as an insert for reference**

strong three-proton signals caused by the methyl and acetate groups lie in the region between 1.0 and 2.5 ppm, together with multiplets caused by certain methylene groups. Most of the protons in the taxane skeleton and the side-chain are observed in the region between 2.5 and 7.0 ppm, and the aromatic proton signals caused by the C-2 benzoate, C-3′ phenyl and C-3′ benzamide groups appear between 7.0 and 8.3 ppm. The characteristic chemical shifts of taxol are shown in Table 5 and the spectrum of taxol are shown in Fig. 6. This fungal taxol has strong cytotoxic activity towards BT220, H116, Int 407, HL 251 and HLK 210 human cancer cells *in vitro*, tested by apoptotic assay and it is indicated that with the increase of taxol concentration from 0.005–0.05 μmol/L, taxol induced increased cell death through apoptosis. With the further increase of taxol concentration from 0.05 μmol/L to 0.5 μmol/L, the taxol-induced cell death through apoptosis only increased slightly. When the taxol concentration was increased from 0.5 μmol/L to 5 μmol/L, the taxol-induced cell death through apoptosis decreased dramatically (Fig.7). Recent accumulating reports have demonstrated that taxol-induced microtubular bundling and mitotic arrest of human leukemia cells are followed by DNA fragmentation and morphological

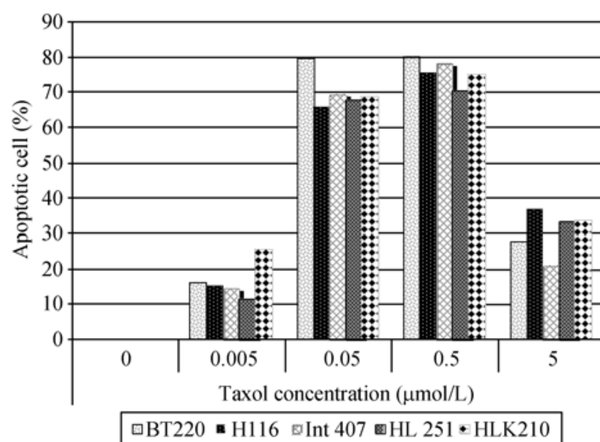


**Fig. 7 Taxol-induced apoptosis by *P. pauciseta* in various cancer cell lines**

features of apoptosis[17–19]. The fungal taxol isolated from this source is spectroscopically identical to standard taxol and accumulates in culture at the level of micrograms per liter. Thus the fungus can serve as a

potential candidate for fungal engineering to improve the production of taxol.

With the discovery that certain endophytic fungi are able to produce taxol has brought the possibility that a cheaper and more widely available product may eventually be available via industrial fermentation. The biggest problem of using fungi fermentation to produce taxol is its very low yield and unstable production. Although the amount of taxol produced by most endophytic fungi associated with Taxus trees is relatively small when compared with that of the trees, the short generation time and high growth rate of fungi make it worth while to continue our investigation of these species.

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## REFERENCES

[1] Sridhar KR, Raviraja NS, Endophytes. A crucial issue. *Curr Sci*, 1995, **69**: 570−571.

[2] Petrini O, Sieber TN, Toti L, *et al*. Ecology, metabolite production and substrate utilization in endophytic fungi. *Nat Toxins*, 1992, **1**: 185−196.

[3] Wani MC, Taylor HL, Wall ME, *et al*. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J Am Chem Soc*, 1971, **93**: 2325−2327.

[4] Georg GI, Chen TT, Ojima I, *et al*. Taxane Anticancer Agents (Basic Science Current Status, American Chemical Society. Symposium Series No. 583). Washington, DC: American Chemicl Society, 1994.

[5] Vidensek N, Lim P, Campbell A, *et al*. Taxol content in bark, wood, root, leaf, twig and seedlings from several *Taxus* species. *J Nat Prod*, 1990, **53**: 1609−1610.

[6] Stierle A, Strobel GA, Stierle D. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of *Pacific yew*. *Science*, 1993, **260**: 214−216.

[7] Stierle A, Stierle D, Strobel G, *et al*. Bioactive metabolites of the endophytic fungi of *Pacific yew*, *Taxus brevifolia*. In: *Taxane anticancer agents* (*Basic Sci*. *Current Status*, American Chemical Society Symposium Series No.583) Edited by Georg GI, TT Chen, I Ojima and DM Vyas. Washington DC). American Chemical Society, 1994, 81−97.

[8] Strobel GA, Hess WM, Ford E, *et al*. Taxol from fungal endophytes and the issue of biodiversity. *J Ind Microbiol*, 1996a, **17**: 417−423.

[9] Li JY, Strobel GA, Sidhu R, *et al*. Endophytic taxol-producing fungi from bald cypress, *Taxodium distichum*. *Microbiology*, 1996, **142**: 2223−2226.

[10] Wang JF, Li GL, Lu HY, *et al*. Taxol from *Tubercularia* sp. strain TF5, an endophytic fungus of *Taxus mairei*. *FEMS Microbiol Lett*, 2000, **193**: 249−253.

[11] Suryanarayanan TS, Kumaresan V, Johnson JA. Foliar fungal endophytes from two species of the mangrove *Rhizophora*. *Can J Microbiol*, 1998, **44**: 1003−1006.

[12] Pinkerton R, Strobel G. Serinol as an activator of toxin production in attenuated cultures of *H. sacchari*. *Proc Natl Acad Sci USA*, 1976, **73**: 4007−4011.

[13] Strobel GA, Yang X, Sears J, *et al*. Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. *Microbiology*, 1996b, **142**: 435−440.

[14] Cardellina JH. HPLC separation of taxol and cephalomannine. *J Chromatogra*, 1991, **14**: 659−665.

[15] Strobel GA. Endophytes as sources of bioactive products. *Micro Infect*, 2003, **5**: 535−544.

[16] McClure TD, Schram KH. The mass spectrometry of Taxol. *J Am Soc Mass Spectrom*, 1992, **3**: 672−679.

[17] Moos PJ, Fitzpatrick PA. Taxanes propagate apoptosis via two cell populations with distinctive cytological and molecular traits. *Cell Growth Differ*, 1998, **9**: 687−697.

[18] Diaz JF, Strobel R, Engelborghs Y, *et al*. Molecular recognition of taxol by microtubules. Kinetics and thermodyamics of binding of fluorescent taxol derivatives to an exposed site. *J Bio Chem*, 2000, **275**: 26265−26276.

[19] Gangadevi V, Muthumary J. Preliminary studies on cytotoxic effect of fungal taxol on cancer cell lines. *Afri J Biotech*, 2007, **6**: 1382−1386.

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