Isolation of *Colletotrichum gloeosporioides,* a novel endophytic taxol-producing fungus from the leaves of a medicinal plant, *Justicia gendarussa*

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Abstract. Taxol is a potent anticancer drug used widely in the treatment of a variety of cancers. An endophytic fungus *Colletotrichum gloeosporioides* (strain JGC-9) was isolated from *Justicia gendarussa,* a medicinal plant 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 163.4 µg/L, thus the fungus can serve as a potential material for fungus engineering to improve the production of taxol. This fungal taxol isolated from the organic extract of this fungal culture also had strong cytotoxic activity towards BT 220, 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 µM, taxol induced increased cell death through apoptosis. This fungus may serve as a potential material for fungal engineering to improve taxol production.

Key words: *Colletotrichum gloeosporioides,* endophytic fungus, medicinal plant, taxol production

Introduction

Studies on endophytic microbes over the past 25 years indicate that they occupy a unique ecological niche and are thought to influence plant distribution, ecology, physiology and biochemistry. 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 (Petrini *et al.* 1992). 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. Endophytic fungi have become a routine part of the natural product screening programs at Merck (Bills & Polishook 1992; Dombrowski *et al.* 1992). It is worthy to note, that of the nearly 300 000 plant species that exist on the earth, each individual plant is host to one or more endophytes. 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, a powerful antimitotic agent with excellent activity against a range of cancers, was originally isolated from *Taxus brevifolia* (Wani *et al*. 1971). Taxol is found in extremely low amounts in the needles, bark and roots (Videnšek *et al*. 1990) and is produced by all yew species (Georg *et al*. 1994). Although complete chemical synthesis of taxol has been achieved, the process is too expensive for commercialization. Presently, all taxol in the world's market has originated from *Taxus* spp. 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 (Stierle *et al*. 1993, 1994), there have been a few reports on the isolation of taxol-producing

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endophytic fungi (Li *et al*. 1996; Strobel *et al*. 1996a; Wang *et al*. 2000), demonstrating that organisms other than *Taxus* sp. could produce taxol. 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. The purpose of this work was to identify this taxol-producing endophytic coelomycetous fungus, *Colletotrichum gloeosporioides* from a medicinal plant, *Justicia gendarussa* Burm. f. (*Acanthaceae*).

Material and Methods

Isolation and identification of endophytic fungi

The fungus used in this study is one of the twenty endophytic fungi isolated from the leaves of medicinal plants in Chennai city, India. The leaf samples were surface sterilized by Suryanarayanan *et al*. (1998). The surface sterilized leaf segments were evenly spaced in Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) medium (amended with chloramphenicol 150 mgl^{-1}). 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 *C. gloeosporioides* strain JGC-9 (MUBL No. 671), 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.

Preparation of fungal extracts

The endophytic fungus was grown in 2 litre Erlenmeyer flasks containing 500 ml of MID medium supplemented with soytone (Pinkerton & Strobel 1976) 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.* (1996b). 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.

Chromatographic separation

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 (Cardellina 1991). 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. Twenty microlitres of the sample was injected each time and detected at 232 nm. The mobile phase was methanol / acetonitrile / water (25:35:40, by vol.) at 1.0 ml min^{-1}. The sample and the mobile phase were filtered through 0.2 µm PVDF filter before entering the column. Taxol was quantified by comparing the peak area of the samples with that of the standard taxol.

Spectroscopic analyses

The purified sample of taxol was analysed 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.

Results and Discussion

Endophytic fungi are increasingly recognized as sources of novel bioactive compounds and secondary metabolites for biological control (Strobel 2003). Therefore, the use of endophytic fungi opens up new areas of biotechnological exploitations, which leads to the necessity of isolation and cultivation of these organisms. The aim 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 *C. gloeosporioides.* Colonies are pale brown or greyish white, consisting of hyaline, septate, branched mycelium. Conidiomata are acervular, separate, composed of hyaline to dark brown septate hyphae. In culture the fungus produces sclerotia, which are dark brown, occasionally setose. Setae are long, brown, septate. Conidiogenous cells are enteroblastic, phialidic, hyaline. Conidia are hyaline, one-

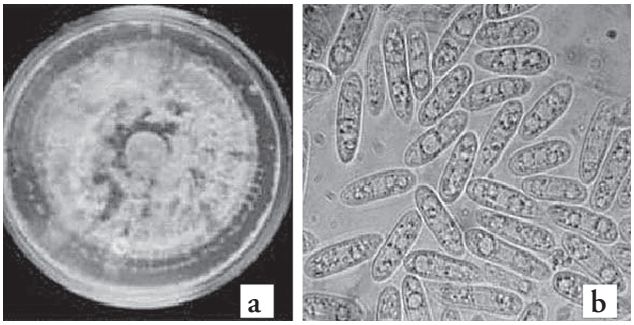

Fig. 1. Growth of the fungus *Colletotrichum gloeosporioides* in Petri dish and conidia of the fungus (top left). **Fig. 2.** High performance liquid chromatogram with UV detection of authentic taxol (a) and fungal taxol from *C. gloeosporioides* (b); the mobile phase was methanol/acetonitrile/water (25:35:40 v/v); retention time of authentic taxol: 10.69 min; retention time of fungal taxol: 10.56 (top right).

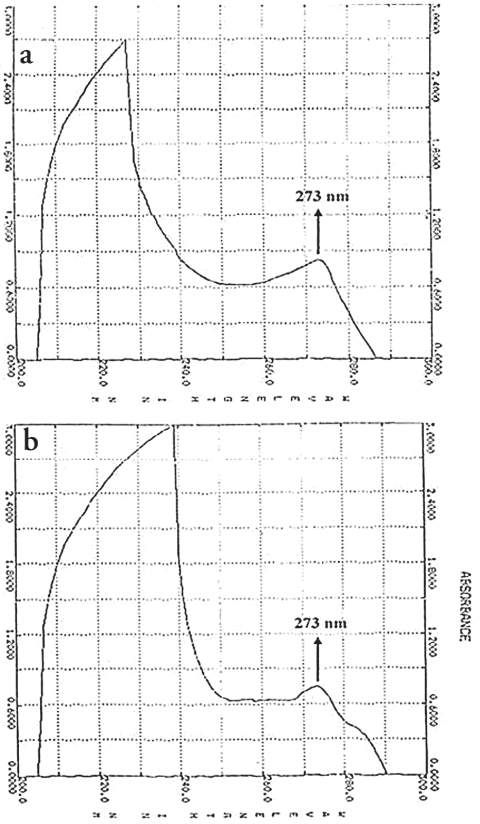

Fig. 3. Ultra Violet absorption spectra of authentic taxol (a) and fungal taxol from *C. gloeosporioides* (b)

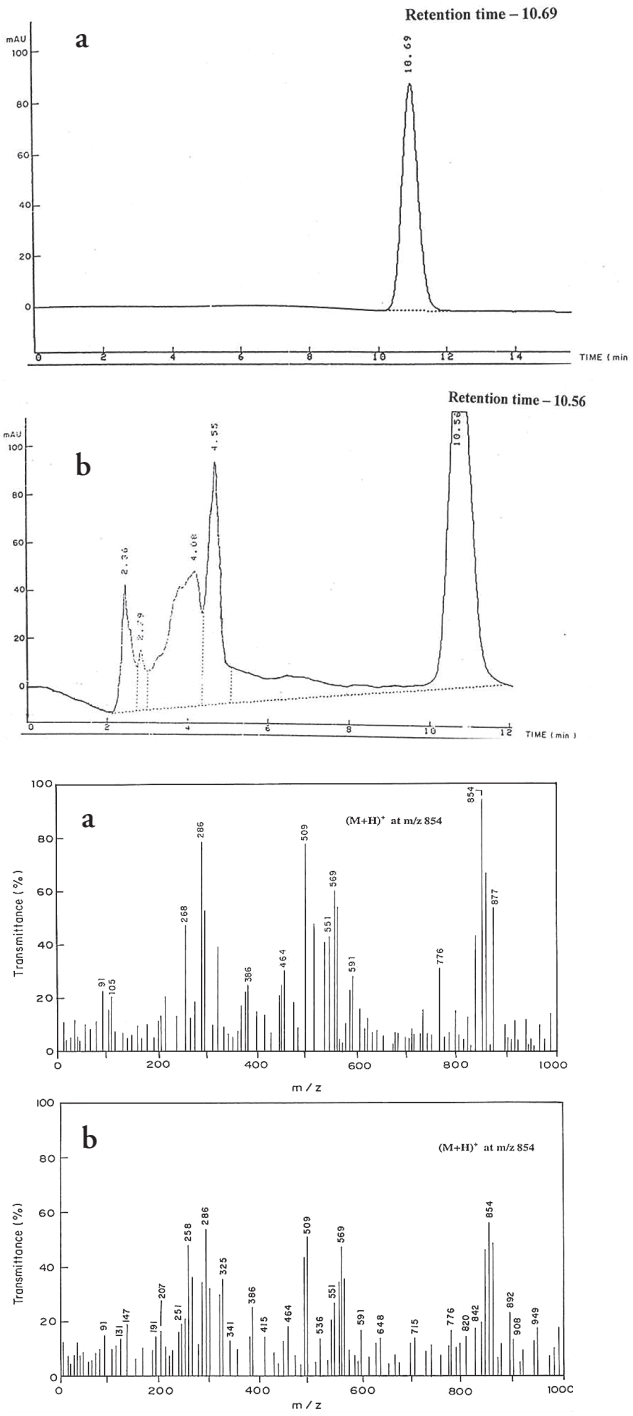

Fig. 4. Fast Atom Bombardment Mass spectra of authentic taxol (a) and fungal taxol from *C. gloeosporioides* (b). The accelerating voltage was 10KV and recorded at room temperature.

celled, straight, cylindrical, obtuse at apices and measuring 9-24 × 3-4.5 µm (Fig. 1). Conidia were used for cultures with the aim to screen taxol production by this fungus. The extract of the fungal culture was examined for the presence of taxol by chromatographic and spectroscopic analyses. The compound has chromatographic properties identical to standard taxol in solvent systems A-E, and gives colour 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 gave a peak with similar retention time as standard taxol (Fig. 2). The presence of taxol in the fungal extract was also confirmed by UV spectroscopy.

The UV spectrum fungal taxol was superimposed on that of standard taxol at 273 nm (Fig. 3). Further convincing spectroscopic evidence for the identity of taxol was confirmed by FAB mass spectroscopy. The FAB mass spectrum of fungal taxol isolated from *C. gloeosporioides* is given in Fig. 4.

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 (McClure & Schram 1992). 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.* (1971), the esterified position was found to be the allylic C_{13} hydroxyl moiety. The amount of taxol produced by this fungus in liquid culture was 163.4 µg/L. Strobel *et al*. (1996b) 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. 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. In this study, the amount of taxol produced by *C. gloeosporioides* was found to be 163.4 µg/L.

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 µM, taxol induced increased cell death through apoptosis. With the further increase of taxol concentration from 0.05 µM to 0.5 µM, the taxol-induced cell death through apoptosis only increased slightly. When the taxol concentration was increased from 0.5 µM to 5 µM, the taxol-induced cell death through apoptosis decreased dramatically (Gangadevi & Muthumary 2007).

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

Bills, G.F. & Polishook, J.D. 1992. Recovery of endophytic fungi from *Chamaecyparis thyoides.* – Sydowia **44**: 1-12.

Cardellina, J.H. 1991. HPLC separation of taxol and cephalomannine. – Journal of Chromatography **14**: 659-665.

Dombrowski, A.W., Bills, G.F., Sabnis, G., Koupal, L., Meyer, R., Ondeyka, J.G., Giacobbe, R.A., Monaghan, R.L. & Lingham R.B. 1992. L-696, 474, a novel cytochalasin as an inhibitor of HIV-1 protease. I. The producing organism and its fermentation. – Journal of Antibiotics **45**: 671-678.

Gangadevi, V. & Muthumary, J. 2007. Preliminary studies on cytotoxic effect of fungal taxol on cancer cell lines. – African Journal of Biotechnology **6**: 1382-1386.

Georg, G.I., Chen, T.T., Ojima, I. & Vyas, D.M. 1994. Taxane anticancer agents. Basic Science Current Status, American Chemical Society. Symposium Series no. 583. American Chemical Society, Washington, DC.

Li, J.Y., Strobel, G.A., Sidhu, R., Hess, W.M. & Ford, E.J. 1996. Endophytic taxol-producing fungi from bald cypress, *Taxodium distichum.* – Microbiology **142**: 2223-2226.

McClure, T.D. & Schram, K.H. 1992. The mass spectrometry of taxol. – Journal of the American Society for Mass Spectrometry **3**: 672-679.

Petrini, O., Sieber, T.N., Toti, L. & Viret, O. 1992. Ecology, metabolite production and substrate utilization in endophytic fungi. – Natural Toxins **1**: 185-196.

Pinkerton, R. & Strobel, G. 1976. Serinol as an activator of toxin production in attenuated cultures of *Helminthosporium sacchari.* – Proceedings of the National Academy of Sciences of the United States of America **73**: 4007-4011.

Stierle, A., Strobel, G.A. & Stierle, D. 1993. Taxol and taxane production by *Taxomyces andreanae,* an endophytic fungus of Pacific Yew. – Science **260**: 214-216.

Stierle, A., Stierle, D., Strobel, G., Bignami, G. & Gothaus, P. 1994. Bioactive metabolites of the endophytic fungi of Pacific yew, *Taxus brevifolia.* – In: G.I. Georg, T.T. Chen, I. Ojima & D.M. Vyas [eds]. Taxane anticancer agents. Basic Science Current Status, American Chemical Society. Symposium Series no. 583. Pp. 81-97. American Chemical Society, Washington, DC.

Strobel, G.A. 2003. Endophytes as sources of bioactive products. – Microbes Infection **5**: 535-544.

Strobel, G.A., Hess, W.M., Ford, E., Sidhu, R.S. & Yang, X. 1996a. Taxol from fungal endophytes and the issue of biodiversity. – Journal of Industrial Microbiology **17**: 417-423.

Strobel, G.A., Yang, X., Sears, J., Kramer, R., Sidhu, R.S. & Hess, W.M. 1996b. Taxol from *Pestalotiopsis microspora,* an endophytic fungus of *Taxus wallachiana.* – Microbiology **142**: 435-440.

Suryanarayanan, T.S., Kumaresan, V. & Johnson, J.A. 1998. Foliar fungal endophytes from two species of the mangrove *Rhizophora.* – Canadian Journal of Microbiology **44**: 1003-1006.

Vidensek, N., Lim, P., Campbell, A. & Clarson, C. 1990. Taxol content in bark, wood, root, leaf, twig and seedlings from several *Taxus* species. – Journal of Natural Products **53**: 1609-1610.

Wang, J.F., Li, G.L., Lu, H.Y., Zheng, Z.H., Huang, Y.J. & Su, W.J. 2000. Taxol from *Tubercularia* sp. strain TF5, an endophytic fungus of *Taxus mairei.* – FEMS Microbiology Letters **193**: 249-253.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P. & McPhail, A.T. 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia.* – Journal of American Chemical Society **93**: 2325-2327.