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Taxol producing fungal endophyte, *Colletotrichum gleospoiroides* (Penz.) from *Tectona grandis* L.

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ABSTRACT

An endophytic fungus, *Colletotrichum gleospoiroides* isolated from leaves of native tree species, *Tectona grandis* was screened for the production of taxol, an anticancer drug. Taxol production was confirmed by methods like Ultra Violet (UV) spectroscopic analysis, Thin layer chromatography and High performance liquid chromatography analysis (HPLC) and the taxol was compared with authentic taxol (Paclitaxel- Sigma grade). The fungal taxol was identical to authentic taxol.

KEY WORDS: *Colletotrichum gleospoiroides,* endophytic fungus, *Tectona grandis,* taxol production

INTRODUCTION

Taxol is a powerful and complex anti-cancer compound that was first isolated from the bark of the Pacific yew, *Taxus brevifolia*. It is a diterpenoid and white to half white crystalline powder containing 11 sterocenters with the empirical formula $C_{47}H_{51}NO_{14.}$ Taxol is a first billion dollar anticancer drug used in the treatment of ovarian, breast and lung cancer and other human tissue proliferating diseases. It can kill tumour cells by enhancing the assembly of microtubules and inhibiting their depolymerisation (Schiff *et al.,* 1979). A major limitation to the therapeutic use of taxol is its very limited resource in nature. However ,this natural resource is threatened day by day due to destructive collection of Taxus bark for taxol. In order to protect Taxus and lighten the pressure of taxol sourcing, other approaches to obtain taxol have been under investigation (Guo *et al.,* 2006). An alternative method of using endophytic fungi for taxol production is in use for the past ten years. Some endophytic fungi belonging to different genera such as *Taxus andreanae, Pestalotiopsis microspora, Alternaria alternate, Periconia* sp.*, Pithomyces* Sp.*, Chaetomella raphigera, Monochaetia* sp. *and Seimatoantlerium nepalense* are reported to produce taxol.

Endophytes are microbes (fungi, bacteria and yeast) that live within the plant tissue without causing any noticeable symptoms of diseases. Endophytes are found in all parts of plants including xylem and Phloem (Petrini,1986). Mainly endophytes are isolated from trees, but only a few herbaceous plants and shrubs had showed the presence of endophytes (Strobel, 2003). Endophytes are capable of synthesizing bioactive compounds that can be used by plants for defense against pathogens and some of these compounds have been proved useful for novel drug discovery. Although known since long time, their importance

become evident only more recently when it was shown that they play specific roles as for instance, protecting the host plant against insect and diseases. The ubiquity of endophytes in the plant kingdom is well established: they have been isolated from all species investigated so far (Arnold *et al*., 2001). Endophytes are also recognized as rich sources of secondary metabolites of multifold importance (Tan & Zou 2001, Strobel & Daisey, 2003). Many new and interesting bioactive metabolites such as antibiotics, antiviral, anticancer and antioxidant compounds which are of pharmaceutical, industrial and agricultural importance have been reported and characterized from fungal endophytes (Tayung *et al.,* 2011). A wide variety of fungi are isolated from the tissues of most terrestrial, aquatic plants ,red and brown algae. Fungi are present in most plant parts, especially the leaves where the tissue is apparently healthy. The fungi may be endophytes, epiphytes or latent pathogens. Endophytes are contained within the plant with or without diseases. Plant tissues remain entire and functional (Pria dharsini *et al.,* 2010).

The present study was aimed at isolation and characterization of taxol from a fungal endophyte, *Colletotrichum gleospoiroides* isolated from leaves of native tree species, *Tectona grandis.*

MATERIALS AND METHODS

Collection of study material

Fresh leaves of *T. grandis* were collected from Boluvampatti forest area, located in 10º59’38’ 56” N latitudes and 76º42’39’32” E longitudes in Western Ghats, Coimbatore district, Tamilnadu. The collected plant material was brought to the Bioprospecting laboratory at Institute of Forest Genetics and Tree Breeding, Coimbatore. The collected leaves were then immediately processed for isolation of endophytic fungi.

Isolation of endophytic fungus

The fungus used in this study is the endophytic fungus isolated from the leaves of *Tectona grandis.* The healthy plant leaves were washed in running tap water and processed as follows: samples were cut into 2mm² segments and were surface sterilized by sequentially dipping into 0.5% Sodium Hypochlorite (2 min) and 70% ethanol (2 min), and rinsed with sterile water, then allowed to surface-dry under sterile conditions(Arnold *et al.,* 2000). The material was then inoculated on to a petridish containing Potato Dextrose Agar (PDA) amended with streptomycin. The petridishes were sealed with Parafilm and incubated at 25±1°C in a light chamber with 12h light followed by 12h of dark cycles and checked from the second day for fungal growth. Individual fungal colonies were transferred onto other plates containing PDA. Fungal spore formation was encouraged by placing the endophytes onto autoclaved *Tectona grandis* leaves. The plates were continuously monitored for spore formation.

Cultivation and metabolic extraction

The fungus was cultivated on potato dextrose broth by placing agar blocks of pure culture (3mm in diameter) of actively growing culture in 2000ml Erlenmeyer flask containing 1500ml media. The flask was incubated in BOD shaking incubator for 30 days at 25ºC with periodic shaking at 150 rpm. The fermented broth of the endophyte was filtered through cheesecloth to remove the mycelia mats. The filtrate was extracted thrice with Ethyl acetate at room temperature. The pooled extract after drying over anhyrdrous $MgSO_4$, was evaporated in a rotary vacuum evaporator. The dry residue so obtained was redissolved in Methanol for further analysis.

Chromatographic separation and spectroscopic analysis

Thin layer chromatography (TLC) analysis

The thin layer chromatography for the fungal sample containing Taxol was carried out on 0.25 mm (10cm x 20 cm) aluminum precoated silica gel plates (Merk) (Cardellina, 1991). Samples were spotted along authentic Taxol (Paclitaxel, SIGMA grade) as internal standard and the plates are developed in solvent (A), chloroform: methanol (7:1,v/v) followed by solvent (B), Chloroform:acetonitrile (7:3. v/v); solvent (C), ethyl acetate: 2- propanol (95:5, v/v) the presence of Taxol was detected with 1% w/v vanillin/sulphuric acid reagent after gentle heating (Cardellina,1991). The RF values of the samples were calculated and compared with authentic taxol.

Ultraviolet (UV) spectroscopic analysis

The presence of taxol in the fungal extract was further confirmed by UV spectroscopy. After the TLC method, the area of plate containing putative taxol was carefully removed by scrapping off the silica at the appropriate Rf and exhaustively eluting it with methanol. After the elution, the crude taxol was performed for the qualitative and quantitative analyses. The taxol samples were analyzed by UV absorption (Hitachi U 2000 Spectrophotometer), dissolved in 100% methanol and compared with authentic taxol (Paclitaxel, Sigma grade). They had a characteristic absorption peak at 235-273nm.

High performance liquid chromatography (HPLC) analysis

Taxol was analyzed by HPLC (Hitachi) using a reverse phase C18 column with a UV detector. A C18 column was used for determining the behavior of the fungal compound by high performance liquid chromatography (HPLC). A 20µl of sample was injected each time and detected at 270nm. The mobile phase was methanol/acetonitrile/water (25:35:40, by vol) at 1.0ml min^{-1} . The sample and the mobile phase were filtered before entering the column. Taxol was quantified by comparing the peak area of the samples with that of the standard taxol. The whole study was carried out during April 2012 to March 2013 at Institute of Forest Genetics and Tree Breeding, Coimbatore.

RESULTS AND DISCUSSION

Endophytic fungi are increasingly recognized as sources of novel bioactive compounds and secondary metabolites for pharmaceuticals (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 fungus from Teak leaves, so that the fungus can serve as a potential material for fungus engineering to improve the production of taxol. The fungus is identified, isolated and screened for Taxol production in Potato Dextrose Broth medium. The extract of the fungal culture was examined for the presence of Taxol by Chromatographic and spectroscopic analysis.

Identification

The morphological identification of endophytic fungal strain was based on the morphology of the fungal culture colony or hyphae, the characteristic of the spores, and reproductive structures if these features are discernible (Wei *et al*., 2007, Carmichael *et al*., 1982 and Barnett *et al.*, 1998). Based on the morphology, the cultures has sparse,

cottony, white to pale grey mycelium with abundant mycelia containing bright orange conidial masses produced on the colonies. (Fig 1) and is identified as *Colletotrichum gleospioroides* (Penz.) (Identifed by Dr. J. Muthumary, Professor (Emeritus), CAS in Botany, University of Madras, Chennai).

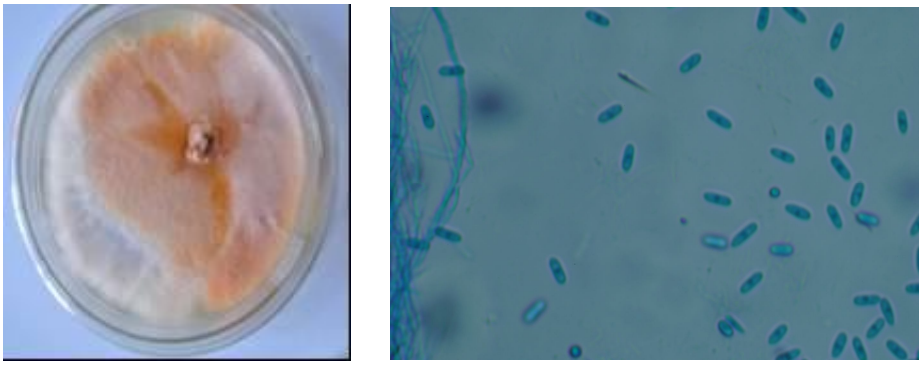

Fig 1: ***Colletrotrichum gloeosporoides***

Mass culture

The identified endophytic fungus was mass cultured in PDA medium, and the extract of fungal culture was examined for the presence of taxol by Chromatographic and spectroscopic analysis.

Extraction

The culture filtrate was harvested after completion of 30 days of incubation period using four layers of cheese cloth to remove the mycelial mat. The filtrate was extracted thrice with ethyl acetate at room temperature. The organic phase was collected and the solvent was then removed by evaporation and the pooled extract after drying over anhydrous $MgSO_4$, was evaporated under reduced pressure at 35°C using rotary vacuum evaporator. The dry solid was re dissolved in methanol for the subsequent separation and extracts were analyzed by Chromatographic separation and spectroscopic analyses.

Thin Layer Chromatography

Thin layer Chromatographic analyses and Ultra Violet (UV) Spectroscopic analysis were carried out for basic screening of Taxol. Taxol, produced by the fungus was detected using a spray reagent consisting of 1% Vanillin (w/v) in sulphuric acid after gentle heating (Cardellina 1991). It appeared as a bluish spot fading to dark grey after 24 hour. The compounds had the same chromatographic properties as that of standard taxol in different solvent systems. It reacts with the spraying reagent and gives colour reaction and their Rf values is same as that of standard taxol. (Fig 2a, Fig 2b). The spectrum was superimposed on that of standard taxol at 261 nm. Hence, the fungus has positive result for the production of taxol (Figs 3a, 3b).

Fig 2 **Fig 3**

TLC analysis: Fig 2 (a) Authentic taxol, (b) C. gloeosporioides – Visible Range
Fig 3 (a) Authentic taxol, (b) C. gloeosporioides – UV Range

High Pressure Liquid Chromatography

The fungal extracts were further analysed by HPLC for the conformation of taxol. The fungal extracts gave a peak when eluting from a reverse phase C18 column, with t the similar retention time as that of standard taxol. (Fig 4a, 4b, 4c, 4d)

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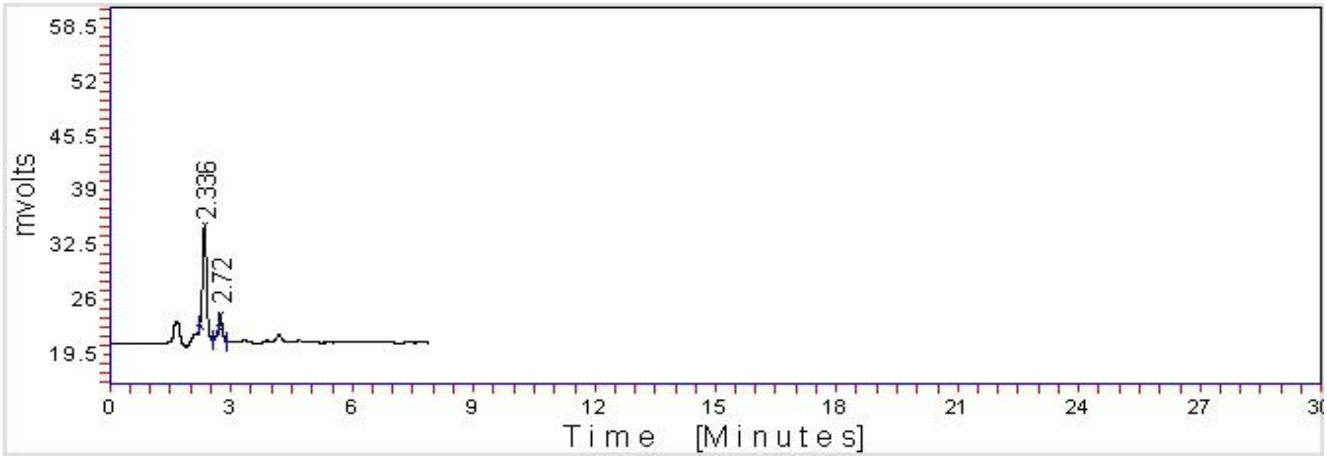

Fig 4a- HPLC analysis of Taxol

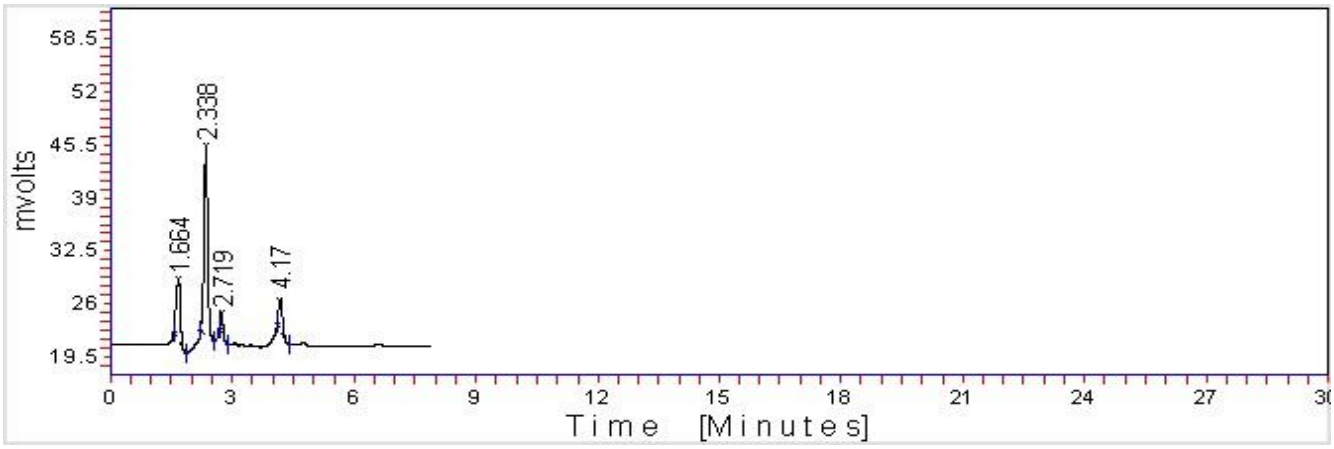

Fig- 4b HPLC analysis for *C.gloeosporiodies* (TLC elucidation Solvent A)

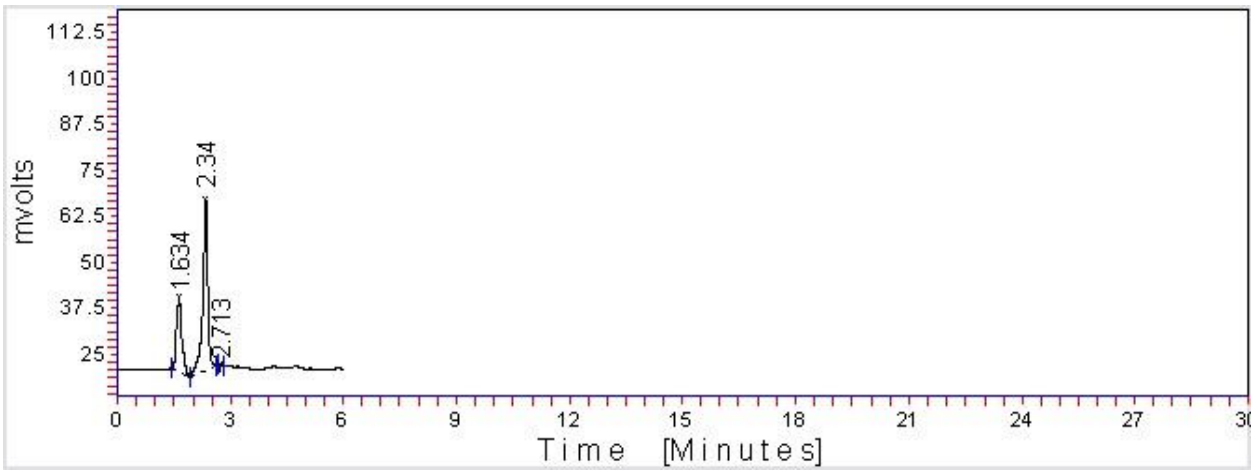

Fig 4c- HPLC analysis for *C.gloeosporiodies* (TLC elucidation Solvent B)

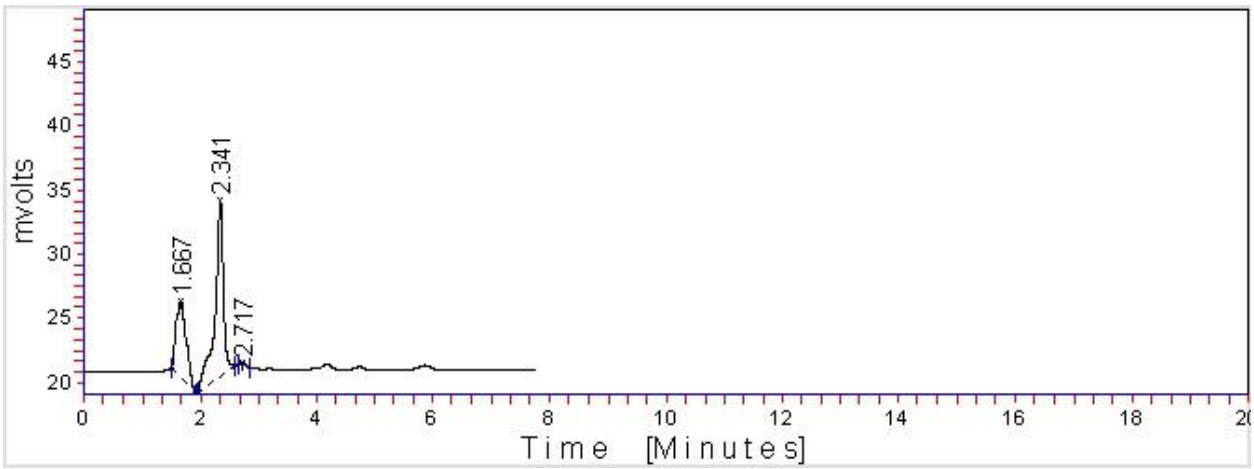

Fig 4d-HPLC analysis for *C.gloeosporiodies* (TLC elucidation Solvent C)

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 to continue with the research in other species. (Gangadevi *et al.*, 2008).

CONCLUSION

C. gloeosporioides, an endophytic fungus which is isolated from teak produces low amount of taxol. It grows well in PDA medium. The significance in the discovery is that the fungus that produces taxol indicate that there are abundant resources of fungi that produce taxol.

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