Abstract: *Plumeria acutifolia* Poiret (Apocynaceae) is grown as an ornamental plant in India, Indonesia, Philippines, and South Africa. Various species of this plant are used as medicine to cure many diseases and also as antipsychotic, diuretic, or antitumour agent. Some iridoids and triterpenoids obtained from this plant are reported to have algicidal, antibacterial and cytotoxic activity. Some species of *Plumeria* are also used for the cure of rheumatism, diarrhoea, blennorhea, venereal disease and leprosy. This study investigates the taxol production by *Colletotrichum gloeosporioides*, an endophytic fungus of *Plumeria acutifolia*. The presence of Taxol was confirmed through TLC, UV and IR spectroscopic methods. The amount of taxol was quantified through HPLC analysis. This is the first report that a coelomyceteous fungus producing taxol from this plant.

**Keywords:** Taxol, *Plumeria acutifolia*, *Colletotrichum gloeosporioides*, endophytic fungi, Coelomycetes.

**Introduction**

Taxus (yew) is a slow-growing evergreen shrub (or) small tree. Taxol (Paclitaxel) is a complicated diterpene alkaloid with anti-tumor activity, which was first isolated from *Taxus brevifolia* by Wani et al. (1971). All taxol used in cancer chemotherapy and scientific research is isolated from yew tree. The mechanism of action of taxol is to inhibit the depolymerization of microtubulin, thus affecting the formation of spindle, prohibiting from mitosis of tumor cell (Zhou et al., 2003). The taxol has been used to cure many malignant tumors, such as breast cancer, ovarian cancer, choriocarcinoma, hysteromyoma (Jones et al., 1996; Pulduinen et al., 1996; Woo et al., 1996). A major limitation to the therapeutic use of taxol is its very limited resource in nature. However, this natural resource is being 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.

Endophytes are microorganisms that reside in the internal tissues of living plants without causing any immediate overt negative effects, are relatively unstudied and potential sources of novel natural products for exploitation in medicine, agriculture and industry. Endophytes are the chemical synthesizers inside the plant’s (Owen et al., 2004). Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against pathogens and some of these compounds have been proven useful for novel drug discovery (Guo et al., 2008).

Anticancer agents from plants currently in clinical use can be categorized into four main classes of compounds: vinca (*Catharanthus*) alkaloids, epipodophyllotoxins, taxanes, and camptothecins. Vinblastine and vincristine were isolated from *Catharanthus roseus* (L.) G. Don (Apocynaceae) (formerly *Vinca rosea* L.) and have been used clinically for over 40 years (Van der Heijden et al., 2004). In our laboratory, research on studies with reference to endophytic fungi and Taxol production has been carried out using several medicinal plants (Bhuvaneswari & Muthumary, 2005; Muthumary & Sasirekha, 2007; Ganagadevi & Muthumary 2007a,b; Gangadevi et al., 2008; Senthil Kumar & Muthumary 2008; Pandi et al., 2009).

*Plumeria*, a plant of the Apocynaceae family is commonly known as “Champa” in India (Gupta et al., 2004). Various species of this plant are used as medicine for the cure of many diseases and used as antipsychotic and diuretic or antitumor agents. *Plumeria* has been investigated in various laboratories for isolation of a variety of iridoides, and triterpenoids exhibiting algicidal, antibacterial cytotoxic and plant growth inhibition activity (Dobhal et al., 2004). Previously there was no report about isolation of taxol from this endophyte. The aim of the present study is to isolate the endophytic fungus *Colletotrichum gloeosporioides* from *Plumeria acutifolia* a latex bearing plant and to study its growth parameter and isolation of taxol from the endophytic fungus.

**Materials and methods**

**Isolation of endophytic fungi**

The fungi used in this study are the endophytic fungi isolated from the leaves of medicinal plant *Plumeria acutifolia* in Guindy Campus Chennai city, India. The healthy plant tissues were washed in running tap water and processed as follows: Samples were cut into 2 mm² 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 PDA (Potato Dextrose Agar) amended with chloramphenicol 150 mg/L medium. The petridishes were sealed using 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 *Plumeria* leaves. The plates were continuously monitored for spore formation by stereo and light microscopy. The identification of the endophytic
fungal- *Colletotrichum gloeosporioides* was based on its morphology and the mechanism of spore production using standard monograph.

**Growth parameters**

**Estimation of radial growth for the fungus:** The radial growth of mycelium of the fungus was measured by growing it on M1D Agar medium. Mycelial discs of 7mm diameter cut from the growing margins of the culture were placed at the centre of the petriplate containing M1D agar medium according to Pinkerton and Strobel (1976). The Petri plates were incubated at 25°C for 10 days. The diameter of the mycelium was measured at daily intervals.

**Estimation of the fungal biomass:** *C. gloeosporioides* was grown in 250ml Erlenmeyer flasks containing 50ml of M1D medium supplemented with 1g soytone 1^{-1} (Pinkerton & Strobel, 1976). The cultures were incubated on different days; i.e. 5, 10, 15, 20, 25 and 30. The mycelial weight (fresh and dry) was recorded at regular intervals.

**Estimation of optimum pH for growth:** The fungus was grown in 250ml Erlenmeyer flasks containing 50ml of MID medium supplemented with 1g soytone 1^{-1} according to Pinkerton and Strobel (1976) and different pH were maintained i.e. 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, and 10.5. After 20 days the fresh weight and dry weight of the mycelium produced per flask was determined.

**Estimation of optimum temperature for growth:** The effect of temperature on vegetative growth was investigated by inoculation a 7-mm disc of mycelium into 50ml of the MID medium according to Pinkerton and Strobel (1976) in 250ml Erlenmeyer flasks. The cultures were incubated in different temperatures i.e. 5, 15, 25 and 35°C. After 20 days the fresh and dry weight of the mycelium produced per flask was determined.

**Effect of carbon sources on the growth:** The fungus was grown in 250ml Erlenmeyer flasks containing 50 ml of M1D broth supplemented with 1g soytone 1^{-1} according to Pinkerton and Strobel (1976) and amended with different carbon sources such as Sucrose, Fructose, Dextrose, Glycerol and Mannitol. The culture was incubated for 20 days. After 20 days fresh and dry weight of the mycelium produced per flask was determined.

**Estimation of taxol production for C. gloeosporioides:** The endophytic fungus was inoculated into M1D medium supplemented with soytone (Pinkerton & Strobel, 1976) and incubated for 21 days. After 3 weeks of static culture at 25 ± 1°C, the culture filtrate was passed through four layers of cheese cloth and extracted with organic solvent (methylene chloride). The extraction and isolation procedure followed was that of Strobel *et al*., 1996(b). 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 TLC 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) ethyl acetate /2-propanol (95:5 v/v); (d) methylene

![Fig 1A. C. gloeosporioides shows maximum growth on 7th day in M1D agar medium](image1)

![Fig 1B. C. gloeosporioides shows maximum growth on 20th day in M1D medium](image2)
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 reverse phase C18 column with a UV detector. Twenty micro litres of the sample was injected each time and detected at 273nm. 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 0.2µm filter before entering the column. Taxol was quantified by comparing the peak area of the samples with that of the standard taxol.

**Spectroscopic analysis**

(a) *UV-Spectroscopic analysis:* The purified sample of taxol was analysed by UV absorption, dissolved in 100% methanol at 273nm in a Beckman DU-40 spectrophotometer and compared with standard taxol.

(b) *Fourier Transformation Infrared (FTIR) Spectroscopic analysis:* The IR spectrum of the compound was recorded on Shimadzu FT IR 8000 series instrument. The purified taxol was ground with IR grade Potassium bromide (KBr) (1:10) pressed in to discs under vacuum using spectra lab pelletiser and compared with authentic taxol. The IR spectra were recorded in the region 4000-400 cm⁻¹.

**Results and discussion**

In this study, extraction of taxol from *Colletrotrichum gloeosporioides* an endophytic fungus of latex bearing medicinal plant *Plumeria acutifolia* (Apocynaceae) is reported. This is the first report that a coelomyceteous fungus producing taxol from this endophyte.

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 grayish white, consisting of hyaline, septate, branched mycelium. The conidiomata are acervular, separate, composed of hyaline to dark brown septate hyphae. In culture the fungus produces sclerotia, which are dark brown, occasionally setose. The setae are long, brown, septate. The conidiogenous cells are enteroblastic, phialidic, hyaline, conidia are hyaline, one celled, straight, cylindrical and obuse at apices and measuring 9-24 X 3-4.5µm. The conidium was used for cultures with the aim of growth studies and to screen taxol production by this fungus. In growth studies, the fungus was found to grow well in M1D agar medium and attained a maximum growth on 7th day (Fig. 1A). Then in M1D broth the fungal mycelium was found to attain a maximum growth on 20th day (Fig. 1B). The optimum pH required for the growth of the fungal mycelium in M1D medium was at 6.5 followed by pH 7.5 (Fig. 1C). The optimum temperature required for the growth of the fungal mycelium in M1D medium was at 25°C (Fig.1D). *C. gloeosporioides* isolated as endophytic fungus from *Artemisia annua* was found to attain a maximum growth of 40-50mm in diameter in PDA medium at 28°C for 5 days (Lu et al., 2000). The carbon source required for the fungal mycelium to attain a maximum growth was found to be dextrose followed by sucrose (Fig.1E). This results matched with the former research on *Fusarium maire* which required glucose for taxol production (Feng Xu et al., 2006) and *Pestalotiopsis spp.* sucrose for sporulation (Ebenezer et al., 2002). The nitrogen source required for the fungal mycelium to attain a maximum growth in the presence of ammonium acetate, followed by beef extract and ammonium tartrate (Fig. 1F). As reported by Feng Xu et al. (2006) *F. maire* requires ammonium nitrate as nitrogen source for the production of taxol. The fungus was incubated in M1D medium for 20 days at 25°C at a pH of 6.5. The extract of the fungal culture was examined for the presence of taxol by chromatographic and spectroscopic analyses. Taxol, produced by the fungus was detected using a spray reagent consisting of

![Fig. 1C. Determination of optimum pH for the growth of *C. gloeosporioides* in M1D medium](http://www.indjst.org)

![Fig. 1D. Determination of optimum temperature for the growth of *C. gloeosporioides* in M1D medium](http://www.indjst.org)
1% vanillin (w/v) in sulphuric acid after gentle heating (Cardellina, 1991). It appeared as a bluish spot fading to dark gray after 24 h. The compound has chromatographic properties identical to standard taxol in solvent systems (a-e), and gives color reaction with the spray reagent and they had Rf values identical to that of standard taxol (Fig. 5). Therefore, it was evident that this fungus showed positive results for taxol production. The fungal sample harvested on 21st day from the media was analyzed by HPLC to further confirm the presence of Taxol. The fungal sample gave a peak when eluting from a reverse phase C18 column, with the similar retention time as authentic Taxol. The quantity of Taxol produced by fungus was calculated based on the area of the sample peak, concentration and peak area of authentic Taxol. This fungus produced 57.54µg/L Taxol in MID liquid medium (Fig. 3A & B). The biggest problem of using fungi in fermentation was less production of taxol, its very low yield and unstable production. The taxol yield of such fungi reported varies from 24ng to 70ng per liter culture (Stierle et al., 1993; Strobel et al., 1996). The amount of taxol produced by the fungus C. gloeosporioides was very low when compared to that of C. gloeosporioides isolated from medicinal plant Justicia gendarussa was found to be 163.4µg/L as reported by Ganga devi and Muthumary (2008). The reason for such a low yield might be due to the milky latex produced by the plant P. acutifolia. The host variation might be a reason for low yield of taxol production. The presence of taxol in the fungal extract was further confirmed by UV spectroscopy. The presence of alcoholic O-group in the compound is evident by its OH stretch at 3448 cm\(^{-1}\). The aliphatic CH- stretch at 2931 cm\(^{-1}\). The C=O stretch positioned 1724 cm\(^{-1}\), where as the amide C=O stretch is shifted to lower value at 1652 cm\(^{-1}\). The instance peak at 1247 16 cm\(^{-1}\)is due to COO stretch. The alkyl C-O stretch of ester is observed at 1072 cm\(^{-1}\). The peak at 707 cm\(^{-1}\) is due to aromatic C, H bond. In extracted sample though the intensity of the bands are very much diminished in the fingerprinting region, appearance of overtone is approximately at 2362 cm\(^{-1}\) (Fig. 4 A & B).

**Conclusion**

An endophytic fungi C. gloeosporioides isolated from the plant P. acutifolia grew well in M1D medium with pH of 6.5 at 25°C and produced taxol in low amount. This has also brought the possibility of producing taxol cheaper and more widely available through protoplast fusion technology in near future.
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