Production of paclitaxel by *Fusarium solani* isolated from *Taxus celebica*

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A fungus was isolated from the stem cuttings of *Taxus celebica*, which produced paclitaxel in liquid-grown cultures. The fungus was identified as *Fusarium solani* based on colony characteristics, morphology of conidia and the 26S rDNA sequence. Paclitaxel was identified by chromatographic and spectroscopic comparison with authentic paclitaxel and its cytotoxic activity towards Jurkat cells *in vitro*.

[Chakravarthi B V S K, Das P, Surendranath K, Karande A A and Jayabaskaran C 2008 Production of paclitaxel by *Fusarium solani* isolated from *Taxus celebica*; *J. Biosci.* **33** 259–267]

1. Introduction

Paclitaxel, a diterpene compound, has been used alone or in combination with other chemotherapeutic agents for the treatment of a variety of cancers, as well as AIDS-related Kaposi sarcoma (Croom 1995; Brown 2003). It was first isolated from the bark of the Pacific yew tree, *Taxus brevifolia* (Wani *et al* 1971), but obtaining paclitaxel from this source requires destruction of trees. The limited supply of the drug has prompted efforts to find alternative sources of paclitaxel. The first report on the isolation of paclitaxel-producing fungi from *Taxus brevifolia* appeared in 1993 (Stierle *et al* 1993; Strobel *et al* 1993). Alternative methods for paclitaxel production, such as chemical synthesis, tissue and cell cultures of the *Taxus* species are expensive and give low yields (Guenard *et al* 1993; Frense 2007). Cost-effective paclitaxel production is possible by industrial fermentation. Several paclitaxel-producing fungi have been identified, such as *Taxomyces andrenae*, *Pestalotiopsis microspora*, *Alternaria* sp., *Fusarium lateritium* and *Periconia* sp. (Strobel *et al* 1996; Li *et al* 1996; Wang *et al* 2000; Zhou 2001; Guo *et al* 2006; Xu *et al* 2006; Yuan *et al* 2006). Among these,

P. microspora isolated from *Taxus wallachiana* produces 60–70 µg/l culture whereas the yield from other species is less than 2 µg/l. Recently, *Fusarium mairie* isolated from China mairie yew yielded 20 µg/l of paclitaxel. This report describes a fungus *Fusarium solani* isolated from *Taxus celebica*, which yields 1.6 µg/l of paclitaxel and its precursors baccatin III and 10-deacetyl baccatin III.

2. Materials and methods

2.1 Isolation of *F. solani* from *T. celebica*

Fresh stem cuttings of *T. celebica* obtained from Bedgeburg, National Pinetum, UK, were defoliated, cut into 2–3 cm nodal segments, surface sterilized by rinsing with 0.1% (w/v) HgCl₂ for 10 min, followed by 5–6 washings with sterile distilled water. The segments were placed in an upright position on MS inorganic salts (Murashige and Skoog 1962) supplemented with vitamins + 100 mg l⁻¹ myoinositol, 1 mg l⁻¹ benzyl adenine, 3% sucrose and solidified with 0.8% agar in 12 × 5.5 cm glass bottles (2–3 nodal cuttings per bottle).

Keywords. Apoptosis; endophytic fungus; *Fusarium solani*; paclitaxel

Abbreviations used: HPLC, high-performance liquid chromatography; ITS, internal transcribed spacer; LC-ESI-MS, liquid chromatography electrospray ionization mass spectra; MALDI, matrix-assisted laser desorption/ionization; PBS, phosphate buffered saline; PCA, potato carrot agar; PCR, polymerase chain reaction; PDA, potato dextrose agar; Rf, Relative front; TLC, thin-layer chromatography

The cultures were incubated at $25\pm 2^{\circ}\text{C}$ in darkness. After several days, fungal mycelia emerged from the cut surface of the segments. The mycelium was transferred to water agar. After 7 days, individual hyphal tips were cut and transferred on either potato dextrose agar (PDA) or MID agar medium (Pinkerton and Strobel 1976). The fungal culture was stored in distilled H_2O at 4°C or in 15% (v/v) glycerol at -70°C as a suspension of spores and mycelium. The isolated fungus was grown in potato carrot agar (PCA) medium for 10 days. The fungal colonies on PCA were white to off-white, floccose, with irregular margins. The reverse side of the culture was cream in colour. The morphology was examined microscopically following staining with cotton blue in lactophenol (figure 1).

2.2 Identification of the fungus

Genomic DNA was isolated from the mycelial mass using methods described elsewhere (Rogers and Bendich 1994).

The internal transcribed spacer (ITS) and D1/D2 26S rDNA regions (~ 1.2 kb) were amplified with pITS1 and pNL4 primers. The amplicons were purified using the Qiagen gel extraction kit. Direct sequencing of the gel-purified polymerase chain reaction (PCR) product was performed with primer NL4 using an ABI Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, USA) (figure 2). Processing of the samples for loading onto an ABI Prism 3130 xl genetic analyser (Applied Biosystems, USA) sequencer was performed according to the manufacturer's instructions. A sequence similarity search was done using Genbank BLASTN (Altschul *et al* 1997).

2.3 Culturing and production of paclitaxel

The fungus was grown in 500 ml of potato dextrose liquid medium in 2 l Erlenmeyer flasks at 25°C in the dark in stationary cultures. After 21 days, 1 l of culture (medium

Figure 1. Culture characteristics of *F. solani*. (A) Mycelium emerging from surface-sterilized stem segments of *Taxus* cultured in Murashige and Skoog medium. (B) A culture growing on the surface of potato dextrose agar. (C) A 21-day-old stationary culture growing in liquid potato dextrose medium. (D) Photomicrograph of conidiating mycelium. (E) One- to 3-celled macroconidia, and unicellular microconidia (arrow).

5'-CACCCGGTGTATTTCGCAGCATCCTTGCGATGCGCGACCTCAGTCCGCCAC
 AGGGTATTACGCAACGG GCTATAACACTCCCCGGAGAGAGCCACGTTCCC
 GATGCCTTTATCCCCCAGGGCGAACTGATGCTGGCCTGAGCCGGAAGAGTG
 CACCGGGGAGAACCCCGGATGATCAACCAAGCCCAAGTCTGGTCACAAGCG
 CTTCCTTTTCAACAATTTACGTACTGTTTAACTCTCTTTTCAAAGTTCTTTTC
 ATCTTTCGATCACTCTACTTGTGCGCTATCGGTCTCTGGCCGGTATTTAGCTTT
 AGAAGACATATACCTCCCATTTAGAGCAGCATTCCCAAACACTACTCGACTCGT
 CGAAGGAGCTTTACAGAGGTTTCGGCGTCCAACCAGACGGGGCTCTCACCCCTC
 TATGGCGTCCCGTTCCAGGGAACCTCGGAAGGCACCTCACCAAAGCATCCTC
 TACAAATTACAACCTCGGGCCCGAGAGCCAGATTTCAAATTTGAGCTGTTGCC
 GCTTCACTCGCCGTTACTGGGGCAATCCCTGTTGGTTTCTTTTCCCTCCGCTTAT
 TGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATTCGAGGTCAACTTCAGA
 AGAGTTGGGTGTTTTACGGCGTGGCCGCGCCGCTCTCCAGTCGCGAGGTGTTA
 GCTACTACGCGATGGAAGCTGCGGCGGGACCGCCACTGTATTTGGGGGGACG
 GCGTGTGCCACGGGGGGCTCCGCCGATCCCCAACGCCAGGCCCGGGGGCCT
 GAGGGTTGTAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGC
 CAATGTGCGTTCAAAGATTTCGATGATTCACTGAATTCTGCAATTCACATTACT
 TATCGCATTTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTT
 GAAGTTTTAATTTAATTTGCTTTGTTTACTCAGAAGATACTAAAAGAAACC
 AGAGTTAATGGTGCGCCTGGCGGGGGCGCCGTTTGTACTGCGACGTCTATTCC
 ATGCCGACCACGTAAGGTAAGTTCACGTGCATTGCATGTGCTGATACACGTA
 TGATACTTCACCTGCACTTCGCATCGGAATCGGCAAAGACGGTGAGGCTACC
 GTAGT-3'

Figure 2. *F. solani* internal transcribed spacer regions (ITS) of rRNA gene cluster, partial sequence and D1/D2 domain of 26S rDNA partial sequence.

plus mycelium) was extracted twice with an equal volume of methylene chloride (Strobel *et al* 1996). The organic phase was taken to dryness under reduced pressure at 35°C. The residue was dissolved in 1 ml methanol.

2.4 Thin-layer chromatography (TLC)

TLC analyses were carried out on Merck 0.25 mm silica gel plates developed in solvents: solvent A, chloroform:methanol (7:1, v/v); solvent B, chloroform:acetonitrile, (7:3, v/v); solvent C, ethylacetate:isopropanol (95:5, v/v); solvent D, chloroform:acetonitrile (4:1, v/v) and solvent E, chloroform:methanol (9.2:0.8, v/v). TLC analysis of the methanolic extract of *F. solani* as well as the methanolic solution of paclitaxel standard was carried out using

solvent E by spotting on the start line of a 0.25 mm (10 × 20 cm) silica gel plate. Paclitaxel 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 which turned dark grey after 24 h. The paclitaxel spot was identified by comigration with standard paclitaxel.

2.5 High-performance liquid chromatography (HPLC)

HPLC separation was performed using a Kromasil C18-column (250 × 4.6 mm) at a flow rate of 1 ml/min. A 20 µl amount of sample was injected. The mobile phase was methanol:water (70:30, v/v). The effluent was monitored at 227 nm (figure 3).

Figure 3. High performance liquid chromatography (HPLC) analysis of the fungal extract (A) and of authentic 10-deacetyl baccatin-III (retention time = 4.3 ± 0.1 min), baccatin-III (retention time = 5.1 ± 0.1 min) and paclitaxel standards (retention time = 15.2 ± 0.1 min) (B). Analysis was performed on a Kromasil 100 C18 $5 \mu\text{m}$ column (4.6×250 mm) using a mobile phase of methanol:water (7:3) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min and absorbance was monitored at 227 nm.

2.6 Spectroscopic analyses

After chromatography, the area with silica gel on the plates containing putative paclitaxel was scraped at the appropriate Relative front (Rf) and exhaustively eluted with methanol

(figure 4). The sample was analysed by matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy. UV-spectrometry was done on the HPLC paclitaxel fraction between 200 nm and 500 nm on a Perkin Elmer (Lambda 35) UV/VIS spectrophotometer (figure 5). Liquid chromato-

graphy electrospray ionization mass spectra (LC-ESI-MS) were recorded on a Bruker Daltonics Esquire 3000 Plus Ion Trap mass spectrometer attached to an Agilent 1000 series HPLC system (figure 6). The samples were infused into the mass spectrometer through a reverse-phase C18 column (UltraPac, 4.0 × 250 mm, LKB, Sweden) by the application of a gradient

elution using a binary solvent delivery system (solvent A – 0.1% formic acid in water and solvent B – 0.1% formic acid in acetonitrile) at a flow rate of 0.3 ml/min. The data were acquired over an m/z range of 500–1000 in positive ion mode and analysed using the Esquire analysis software (figure 7).

Figure 4. Thin-layer chromatography (TLC) analysis of fungal paclitaxel. Samples were subjected to TLC on a silica (silica 60 F₂₅₄) plate. The chromatogram was developed using the solvents CHCl₃:CH₃OH (9.2:0.8, v/v) and sprayed with 1% vanillin–sulphuric acid to detect paclitaxel. Lane 1: methanolic solution of paclitaxel standard; lanes 2, 3 and 4 are methanolic extracts of *F. solani*.

Figure 5. UV absorption spectrum of the peak of the high performance liquid chromatography (HPLC) corresponding to 15.2 min in figure 3A. Dotted line, fungal paclitaxel; solid line, standard paclitaxel.

Figure 6. Matrix-assisted laser desorption/ionization mass spectra (MALDI-MS) of thin-layer chromatography (TLC) purified fungal paclitaxel. Note the characteristic peaks in the spectrum at the molecular ion plus H^+ = 854 and the molecular ion plus Na^+ = 876.

2.7 Apoptosis assay and FACS analysis

As described elsewhere (Fraker *et al* 1994), Jurkat cells (1×10^6 cells/ml) were treated with either $10 \mu\text{M}$ fungal compound or $10 \mu\text{M}$ standard paclitaxel in RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS) for 24 h. The cells were centrifuged at 300 g for 5 min, resuspended in $100 \mu\text{l}$ of 50 mM phosphate buffered saline (PBS) and fixed with ice-cold 70% (v/v) ethanol for 30 min at -20°C . The cells were pelleted and washed once with PBS and stained with ethidium bromide ($50 \mu\text{g/ml}$) staining solution containing $100 \mu\text{g/ml}$ RNase A and 1% (v/v) Triton

X-100 for 60 min at 37°C . The cells were then analysed by a fluorescence-activated flow cytometer (FACScan, Becton Dickinson, USA) (figure 8).

3. Results and discussion

3.1 Identity of fungus isolated from *T. celebica* stem segment

As shown in figure 1, a white mycelium proliferated into the air from the cut surface of the surface-sterilized

Figure 7. Liquid chromatography electrospray ionization mass spectra (LC-ESI-MS) analysis of crude methylene chloride extract of *F. solani*. (A) HPLC separation of the compounds from the *Fusarium solani* culture extract. (B) ESI-MS spectrum obtained in the full scan mode recorded at a cone voltage of 60 V, showing 10-deacetyl baccatin III ($\text{M}+\text{NH}_4^+=561$), baccatin III ($\text{M}+\text{NH}_4^+=603$) and paclitaxel [$\text{M}+\text{H}^+=854$ & $\text{M}+\text{K}^+=893$] (arrows).

Figure 8. Apoptotic activity of fungal paclitaxel through FACS analysis. Jurkat cells were cultured with 10 μ M of the fungal paclitaxel for 24 h. The cells were then fixed in ethanol, stained with ethidium bromide and the percentage of apoptotic population was determined by FACS analysis. (A), (B) and (C): FACS profile; (D) Histogram of the FACS profile. Standard paclitaxel (10 μ M) was taken as the positive control and only DMSO as the vehicle control.

stem segment. Since the mycelium growth emerged from surface-sterilized stem segments, this growth was assumed to be that of an endophytic fungus. We did not confirm the endophytic nature of the mycelium by microscopic examination of tissue sections. The fungus was identified after induced sporulation in a starvation medium. The fungus grew rapidly in PCA medium at 25°C. The identity of the fungus was determined by Dr S K Singh of the Agharkar Research Institute, Pune and by Dr G S Prasad of the Microbial type culture collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The culture produced macroconidia on slender, 85 μ m long phialides. The macroconidia were 25–40 $\times$ 3.75 μ m. The culture also produced round or oval microconidia (figure 1).

Analysis of the ITS and D1/D2 26S rDNA sequence revealed 99% identity with *Fusarium solani* voucher NJM 0271. By the morphology of the fungal spores and 26S rDNA sequencing (figure 2), the fungus was identified as *F. solani*. This fungus is the different from the previously reported endophytic paclitaxel-producing species of *Fusarium*.

3.2 Paclitaxel production

Paclitaxel was identified based on TLC, HPLC and spectral analyses by UV, MALDI-MS and LC-ESI-MS. Figure 3 shows the HPLC chromatogram of the crude extract. Separation of the mixture of various taxanes in the extract was done with methanol:water (70:30 v/v) over a period of 30 min (figure 3A). Standards of the three most abundant taxanes – baccatin III, 10-deacetyl baccatin III and paclitaxel – were resolved by this system as shown in figure 3B. Baccatin III, 10-deacetyl baccatin III and paclitaxel were identified in the crude extract by HPLC by comparison of retention time to standard peaks. Figure 4 shows the TLC analysis of the crude fungal extract. One of the fungal compounds exhibited the same R_f values as authentic paclitaxel, yielding a characteristic blue spot with vanillin–sulphuric acid reagent.

Figure 5 shows the UV spectrum of the HPLC fractions of the paclitaxel. The isolated compound had a UV spectrum similar to authentic paclitaxel, with a maximum absorbance at 227 nm. MALDI-MS was used as an independent method

for identification of paclitaxel. In the TLC purified sample, the molecular ions ($m/Z = 854$ of $M+H^+$) and ($m/Z = 876$ of $M + Na^+$) corresponding to paclitaxel were present as shown in figure 6. A full-scan LC-ESI-MS chromatogram for a crude extract is shown in figure 7. The presence of molecular ion ($m/Z = 854$) at the appropriate retention time confirmed that the compound was paclitaxel. The concentration of paclitaxel analysed by HPLC was $1.6 \mu\text{g/l}$.

3.3 Apoptotic activity of fungal paclitaxel

We determined whether the paclitaxel purified from *F. solani* exhibits apoptotic activity. Jurkat cells were cultured with the fungal paclitaxel for 24 h followed by fixing the cells, staining with ethidium bromide and estimating the apoptotic cells by FACS analyses. As seen from figure 8, the fungal paclitaxel induced apoptosis in 66% of cells as compared with standard paclitaxel, which exhibited apoptotic activity in 51% of cells.

4. Conclusion

The *F. solani* described here is different from previously reported endophytic paclitaxel-producing fungi. Besides paclitaxel, the fungus also yielded taxanes, baccatin III and 10-deacetyl baccatin III, which are important intermediates for paclitaxel and semisynthesis of paclitaxel in industry. Knowledge of the biosynthesis of paclitaxel in fungi is necessary for determining the conditions for fermentation and metabolic engineering of the strains for overproduction of paclitaxel. We cloned a partial taxane 13 α hydroxylase cDNA (Gen bank accession No. EF626531) from this fungus. The amount of paclitaxel produced by *F. solani* is low. Quantification by HPLC indicated that $1.6 \mu\text{g}$ of paclitaxel was produced per litre of culture. The optimum culture conditions for paclitaxel production are under further study. Improved culturing techniques and the application of genetic engineering may improve paclitaxel production by *F. solani*.

Acknowledgements

Thanks are due to Mr Daniel Luscombe, Bedgebury for sending fresh stem cuttings of *Taxus celebica*. We are grateful to Professor Ramesh Maheshwari for useful suggestions and critical reading of the manuscript. We thank Professor Sunil K Podder for helpful discussions. We also thank Ms Akhila T S for her technical assistance. We are thankful to Professor P K Das and K R Prasad for allowing us to use their laboratory facilities. We thank Dr Omana Joy and Vamsi for the FACS analysis. This work was supported by a grant from the Department of Biotechnology, New Delhi. BVSKC is a recipient of a research fellowship from the Council of Scientific and Industrial Research, New Delhi.

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MS received 4 October 2007; accepted 15 February 2008

ePublication: 28 March 2008

Corresponding editor: RITA MULHERKAR