JMB 2008; 27 (4) DOI: 10.2478/v10011-008-0022-3

UDK 577.1 : 61 ISSN 1452-8258

JMB 27: 454–458, 2008

Original paper
Originalni naučni rad

TAXOL PRODUCTION BY FUSARIUM ARTHROSPORIOIDES ISOLATED FROM YEW, TAXUS CUSPIDATA

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Summary: A taxol-producing endophyte was *Fusarium arthrosporioides*. As a novel taxol resource, a taxol-producing endophyte was successfully isolated from the yew tree, *Taxus cuspidata* Sieb. et Zucc. According to the morphological characterization and the ITS4-ITS5 sequences, the isolated endophytic fungus was identified as *Fusarium arthrosporioides*. Fermentation conditions for taxol production were optimized with the isolated strain (F-40) of *F. arthrosporioides*. The fungal taxol was analytically confirmed by TLC, RP-HPLC, LC-MS and NMR. *F. arthrosporioides* isolated from yew was found to produce taxol with a maximum yield of 131 μg/L. Precise methods were established for detecting the fungal taxol and its derivatives.

Key words: anticancer, endophytic fungus, *Fusarium arthrosporioides*, taxol

Kratak sadržaj: Korišćena endofita koja proizvodi taksol bila je *Fusarium arthrosporioides*. Kao novi izvor taksola, ta endofita koja proizvodi taksol uspešno je izolovana iz stabla tise, *Taxus cuspidata* Sieb. et Zucc. Prema morfološkoj karakterizaciji i sekvencama ITS4-ITS5, izolovana endofitna gljiva identifikovana je kao *Fusarium arthrosporioides*. Uslovi fermentacije za proizvodnju taksola poboljšani su pomoću izolovanog niza (F-40) *F. arthrosporioides*. Taksol dobijen iz gljive analitički je potvrđen pomoću TLC, RP-HPLC, LC-MS i NMR. Utvrđeno je da *F. arthrosporioides* izolovana iz tise proizvodi taksol u količini od maksimalno 131 μg/L. Ustanovljene su precizne metode za otkrivanje taksola i njegovih derivativa u gljivama.

Ključne reči: antikancer, endofitna gljiva, *Fusarium arthrosporioides*, taksol

Introduction

The resource of Taxus trees worldwide is very limited (1), and recovery of taxol from the tree bark generally means fatal damage to the trees. Taxol content in the bark is quite low (< 0.02% by weight). About 1000 kg of bark are needed to produce 1 kg of taxol, and other similar compounds such as taxine (the taxane diterpenoid with a basic side chain at C-5) and taxinine (taxane diterpenoid with a C-5 cinnamoyl group) that are co-extracted with taxol. The growth rate of Taxus trees is very slow, it usually takes about 20 years for them to reach a height of 4.5 metres. Therefore, finding other sources of taxol has been a research topic of extensive interest. Since 1993, several endophytic fungi, such as *Taxomyces andreanae* and *Pestalotiopsis microspore*, etc., have been reported to produce taxol (2, 3). Thus, organisms other than Taxus sp. produce taxol, but only at low levels, 24–1081 ng/L (2–5). Here we report a taxol-producing *Fusarium arthrosporioides* with a yield of 131 μg/L.

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Materials and Methods

Isolation and identification of endophytic fungi from T. cuspidate

The fungus used in this study was one of 41 endophytic fungi isolated from the inner bark of *T. cuspidata* obtained in Kang-Won province, Korea. The microorganisms were isolated as follows: bark was washed with sterilized Tween-80 for an hour. Removed epidermis, tissue obtained from phloem and xylem were cut into small pieces (0·5×0·5 cm), and then were treated with 70% (v/v) ethanol for 15 seconds. These sterilized fragments were then treated with sodi-

um hypochlorite for 15 mins. Treated tissue was damp-dried on absorbent paper towelling before being placed on potato dextrose agar (PDA) to decrease the amount of bacterial contamination. In a second procedure, the outer bark was removed with a sterilized sharp blade. Small pieces of inner bark were placed on the surface of PDA in Petri plates. After several days, fungi were observed growing from the inner bark fragments on the plates. Individual hyphal tips of the various fungi were removed from the agar plates and placed on a new PDA medium, and incubated at 25 °C for at least 2 weeks (6). Fungal identification methods were based on a the morphology of the fungal culture, the mechanism of spore production and the characteristics of the spores (7, 8).

Fungal hyphae were transferred to a fungal growth medium, and 10 successive cultures were carried out.

Screening of taxol producing endophytes

The isolated fungal strains were incubated at 25 °C for 21 days in 500 mL Erlenmeyer flasks containing 200 mL potato dextrose liquid medium in shake incubator, shaking speed was 150 rpm. The entire culture was blended well, mycelia were ground thoroughly, then extracted with equal volumes of a mixture of chloroform/methanol (20:1, v/v), and the organic phase was evaporated *in vacuo* to dryness at 40 °C. The residue was dissolved with 1 mL methanol, and subjected to thin layer chromatography (TLC) on a plate (10×10 cm, Silica Gel60, F254, Merck, Germany) developed in solvent system A: chloroform/methanol (7:1, v/v), B: chloroform/acetonitrile (7:3, v/v), C: ethyl acetate/isopropanol (95:5, v/v), D: methylene dichloride/tetrahydro-furan (6:2, v/v).

Identification by ITS sequence comparison

ITS5/ITS4 rDNA partial-sequencing and phylogenetic analyses were conducted (9). The chromosomal DNA was isolated by following Kim et al. (10). ITS4/ITS5 primers were designed and named according to White et al. (11). The amplification profile was 30 cycles of 95 °C for 30 s, 42 °C for 60 s, 72 °C for 60 s, preceded by an initial denaturation at 95 °C for 90 s and followed by a final extension at 72 °C for 180s. The PCR products were purified using the QIAquick PCR purification kit (Qiagen). The purified PCR product was sequenced using the ABI PRISM BigDye Terminator cycle sequencing kit and an Applied Biosystems model 310 automatic DNA sequencer (Applied Biosystems). The sequence data were assembled using the SeqMan DNASTARTm (Madison, WI) and were compared with available rRNA sequences from GenBank using the BLAST program to determine the approximate phylogenetic affiliation.

Preparation of taxol from the culture of F-40

The residue extracted from liquid culture was dissolved in 1 mL methanol, and subjected to thin layer chromatography (TLC) on a 0·25 mm (10×20 cm) silica gel plate developed in solvent system A (chloroform/ methanol 7:1, v/v) against authentic taxol as control. After chromatography, the area of plate containing putative taxol was carefully removed by scraping off the silica at the appropriate Rf, and exhaustively eluting it with methanol. The elution was then used for the subsequent identification steps.

HPLC, UV, MS and H^1-NMR

A C18 column (5×300 mm, Waters) was used for determining the behaviour of the fungal compound by high performance liquid chromatography (HPLC, Beckman, USA). Samples in 10 μL of methanol were injected and elution was done with methanol/H2O (65:35, v/v) at room temperature. A variable wavelength recorder set at 227 nm was used to detect compounds eluting from the column.

MS was done on taxol samples with a Finnigan LC-MS (Hewlett-Packard Co., Palo Alto, CA). The sample was dissolved in methanol and injected with a spray flow of 2 μL min^{-1} and a spray voltage of 2·2 kV. ^{1}H-NMR spectra were obtained on a Bruker (DRX 300-MHz, Japan) using CDCl3 as solvent.

Results

Identification of strain F-40

The screened strain of F-40 grew fast, reaching 7·5-8·0 cm in diameter after 4 days culture at 26 °C on PDA. Aerial mycelium was abundant, loosely fluffy to cottony, whitish with a dash of rose. Chlamydospores generally abundant in hyphae. The ITS4/ITS5 rDNA sequence of strain F-40 has been deposited in GenBank under the accession number AY575714. In a BLAST analysis, the determined ITS4/ITS5 rDNA sequence of strain F-40 showed 99·59% homology with that of *F. arthrosporioides*, 92·82% with that of *F. redolens*. A dendrogram constructed of the ITS4/ITS5 rDNA sequences of selected *Fusarium spp*. also showed that there was a close phylogenetic relationship between F-40 and *F. arthrosporioides* (*Figure 1*), this suggested that the strain F-40 was *F. arthrosporioides*.

Fungal taxol from the F-40 culture

Eight compounds named compound 3-1, 3-2, 3-3, 3-4, 3-5, 6-1, 6-2, 6-3 were obtained by prepared TLC. A fungal compound 3-5 having TLC properties comparable to taxol in solvent systems A, B, C, and D, giving the same colour reactions with seven different reagents on TLC plate (12) for visualization was consis-

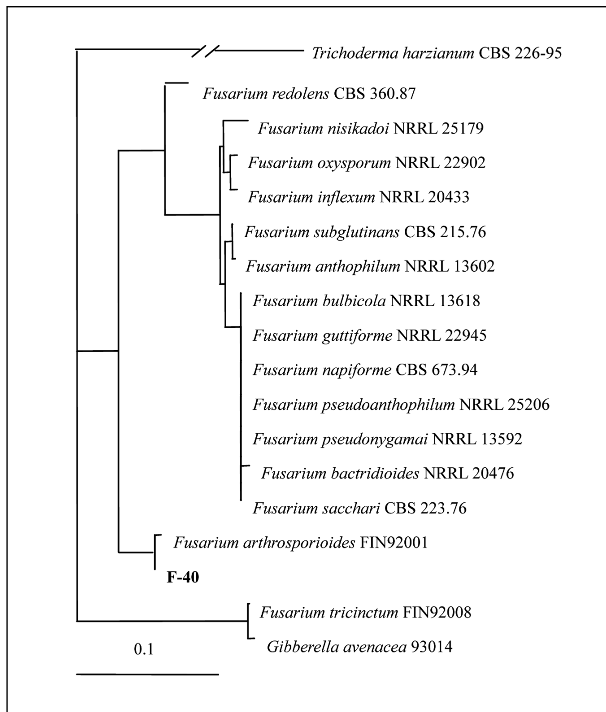

Figure 1 Phylogenetic tree showing the relationship of Strain F-40 to members of the genus *Fusarium* and related taxa.

tently isolated from the F-40 culture, the same Rf and colors found were shown between sample and the authentic taxol on TLC plate. The fungal compound isolated from F-40 yielded a UV absorption spectrum that was similar to authentic taxol, with a maximum at 227 nm (*Figure 2*) by HPLC. It could be seen that 227 nm represents the maximum absorbance wavelength for the UV spectrum of taxanes, whereas 276 nm was the λmax of taxines. We also observed that the fungal compound gave a single peak when eluting from the C_{18} HPLC column, with about the same retention time as authentic taxol, and the recovery of taxol is 97·5%. The standard curve of taxol:

$$\text{Amount}=6{\cdot}79984e^{-005}\times\text{Area}-27{\cdot}6506,$$
$$R^2=0{\cdot}995265,\ \text{Amount}_{min}=0{\cdot}4\ \mu g.$$

131 μg/L of taxol produced by F40 was measured with the standard curve of taxol in the 13-18 days culture in the best fermentation conditions. When authentic taxol was co-chromatographed with the fungal compound, only a single peak appeared, having a retention time of 18.20 min. Further convincing spectroscopic evidence for the identity of taxol was obtained by the electrospray mass spectroscopy. Characteristically, authentic taxol yielded a $[M+Na]^+$ peak at 876 nm (6, 13). Extraction gave some LC signals using a 15 cm column, at 6.6 min for compound 3-5 and at 12.4

Figure 2 UV spectrum on various peak of HPLC, δ the UV spectrum of A~F, II HPLC chromatogram of peak A~F.

min for compound 6-3, the typical molecular ions $[M+Na]^+$ associated with compound 3-5 (*Figure 3*) and compound 6-3 (*Figure 4*) are m/z 876. ^{1}H-NMR chemical shifts of compound 3-5 were seen (*Figure 5*). These results confirm that the fungal compound 3-5 is taxol. Compound 6-3 is concluded as 7-epi-taxol.

Discussion

The these primers used to amplify the ITS region were ITS4 and ITS5 which typically amplify a region between 600~800 bps oligonucleotide primers generated DNA sequences of the expected size for the entire ITS region, from all the fungi present in our fungal samples. Even though the ITS4/ITS5 rDNA of the genus *Fusarium* has highly conserved regions, some regions of its were variable, therefore, the ITS4/ITS5 rDNA sequence were compared as a powerful tool for deducing the phylogenetic relationships within the *Fusarium* genus. The ITS4/ITS5 rDNA sequences were obtained and were then compared with the known sequence.

UV spectra offered an alternative for the cost-effective separation of taxanes and taxines in complex *F. arthrosporioides* extracts, a clearer prospect of the corresponding spectra is shown in *Figure 2*. More than 4 taxines were detected by the photodiode array detector (*Figure 2*). Since has been suggested that taxol be produced with these compounds, these compounds could serve as alternative starting material for semi-synthetic production of taxol or taxol derivatives.

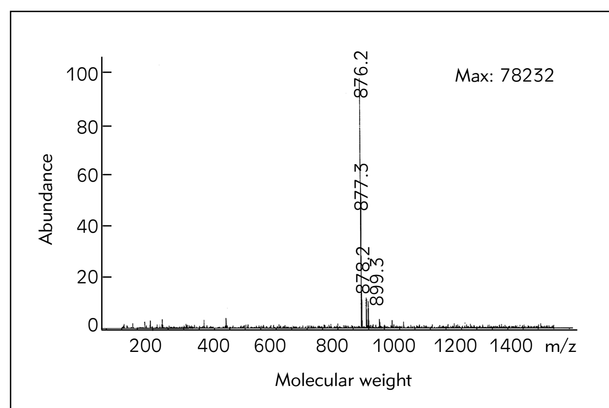

Figure 3 Assay of compound 3-5 by LC-MS. The main peaks given in the spectrum include the ions produced by molecular-related ion m/z 876 $[M+Na]^+$.

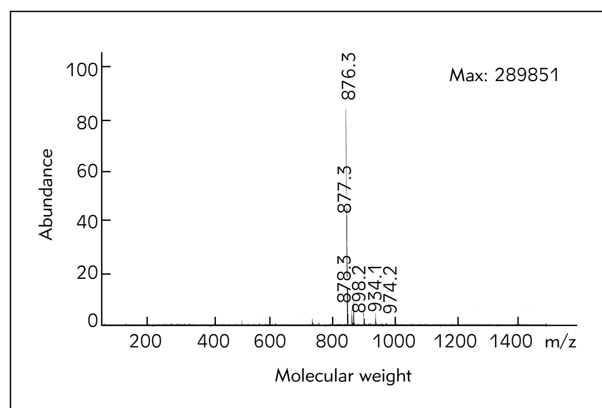

Figure 4 LC-MS analysis of compound 6—3, the main peaks given in the spectrum include the ions produced by molecular-related ion m/z 876 $[M+Na]^+$.

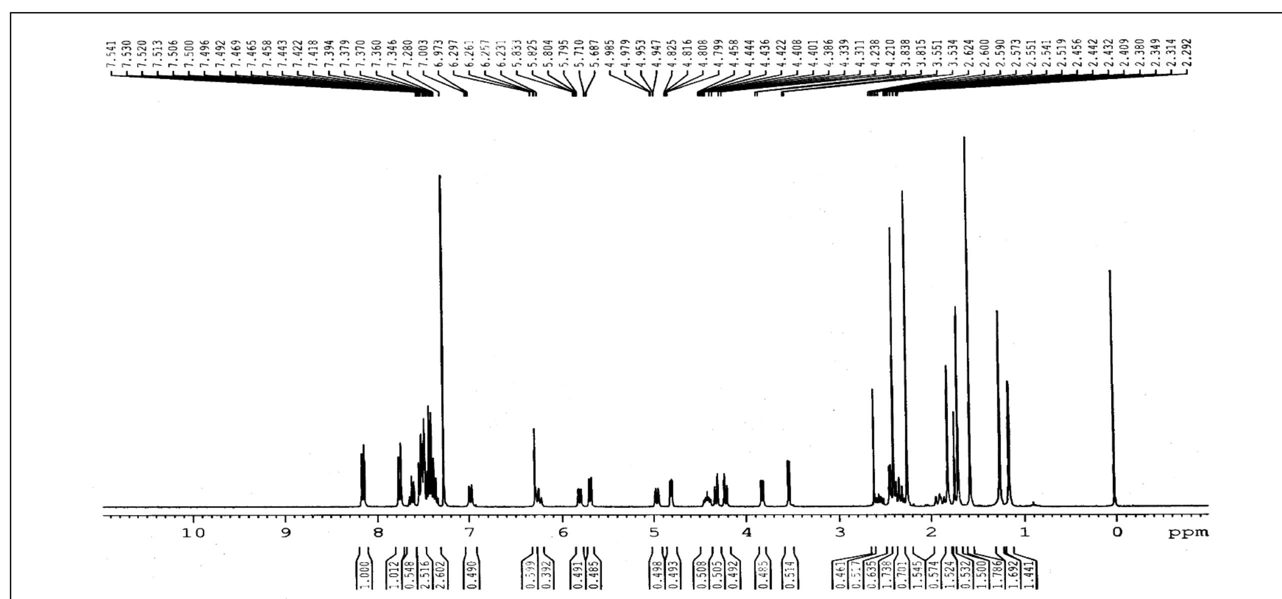

Figure 5 ^{1}H-NMR of compound 3-5 δ: 1·16(3H,s), 1·25(3H, s), 1·69 (3H, s), 1·81 (3H, d, J=1·1), 2·29, 2·40 (each 3H, s), 1·86 (1H, m), 2·34 (2H, m), 2·55 (1H, ddd, J=6·6, 9·7, 14·9), 3·5(1H, d, J=7·0), 3·8 (1H, d,J=7·0),4·2, 4·3 (each 1H, d, J=8·5), 6·24 (1H, brt, J=8·3), 6·27 (1H, s), 6·96 (1H, d, J=8·8), 7·4 (5H, m), 7·61 (2 H, t, J=7), 7·74 (2H, d, J=7), 8·14 (2H, d, J=7) .

Through HPLC and TLC four fungal isolates (A-21, F-35, X-38 and F-40) were isolated then screened for taxol production. Strains A-21, F-35 and X-38 produced low taxol concentrations and are being further researched in our lab. According to these results, taxol-producing endophytes were mainly produced from bark, although *Pestalotiopsis* sp. had been reported from the soil of yew. Up to now, 131 μg /L of taxol was produced in *F. arthrosporioides*, *F. arthrosporioides* strain F-40 and the potential market for this strain is under further study.

Acknowledgements. This project was supported by the Science Foundations of Jilin (No.20070903-01).

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Received: March 18, 2008

Accepted: May 21, 2008