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Original/*Otros*

Isolation and identification of an endophytic fungus producing paclitaxel from *Taxus wallichiana* var *mairei*

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Abstract

The objective of this study was to isolate endophytic fungi producing paclitaxel from yew for the purpose of paclitaxel manufacture. Surface sterilized bark of *Taxus wallichiana* var. *mairei* was used as source material and potato dextrose agar culture medium was used in isolation of endophytic fungi. Fungal cultures were extracted with a mixture of chloroform / methanol (1:1, v/v) and the paclitaxel in the extracts was determined and authenticated with LC-MS. An endophytic fungus that produced paclitaxel was identified by ITS rDNA and 26S D1/D2 rDNA sequencing. The results showed that a total of 435 endophytic fungal strains were isolated from *T. wallichiana* var. *mairei* and purified. Only one of these strains produced paclitaxel and it belongs to *Fusarium*. The paclitaxel productivity in whole PDB culture and that in spent culture medium from this strain is 0.0153 mg/L and 0.0119 mg/L respectively. The paclitaxel content in dry mycelium is 0.27 mg/kg. This isolated endophytic fungus produced paclitaxel at a considerable level and shows potentiality as a producing strain for paclitaxel manufacture after strain improvement.

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Key words: Taxus wallichiana *var.* mairei. *Paclitaxel*. *Endophytic fungus*. Fusarium.

AISLAMIENTO E IDENTIFICACIÓN DE UNA PRODUCCIÓN DE HONGOS ENDÓFITOS DE PACLITAXEL *TAXUS WALLICHIANA* VAR. *MAIREI*

Resumen

El objetivo de este estudio fue aislar hongos endofíticos productores de paclitaxel a partir de tejo con el propósito de fabricar paclitaxel. Se utilizó la superficie de la corteza esterilizada de *Taxus wallichiana* var. *mairei* como material de origen y dextrosa de patata en medio de cultivo de agar para el aislamiento de hongos endófitos. Los cultivos de hongos se extrajeron con una mezcla de cloroformo/metanol (1:1, v/v) y el paclitaxel en los extractos se determinó y autentificó con LC-MS. Un hongo endófito que produjo paclitaxel fue identificado por su ADNr 26S y secuenciación D1/D2 ADNr. Los resultados mostraron que un total de 435 cepas de hongos endófitos se aislaron y purificarón a partir de *T. wallichiana* var. *mairei.* Solo una de estas cepas produce paclitaxel y pertenece a *Fusarium.* La productividad del cultivo de paclitaxel procedente de esta cepa es 0,0153 mg/L y 0,0119 mg/L, respectivamente. El contenido de paclitaxel en micelio seco es 0,27 mg/kg. Este aislado de hongos endófitos produjo paclitaxel a un nivel considerable y muestra potencial como cepa para la fabricación de paclitaxel después de llevar a cabo una mejora de las cepas.

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Palabras clave: *Taxus wallichiana var. Mairei*. *Paclitaxel*. *Hongos endofítico*. *Fusarium*.

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Introduction

Paclitaxel has high activity as an anticancer agent and is widely used in hospitals and clinics. Paclitaxel is a naturally occurring chemical component that was first identified from the bark of yew trees, but presents at low levels[1]. In recent years, lots of yew trees have been destroyed or seriously damaged by harvesting to extract paclitaxel. Although some fungi endophytic to yew have been found to produce paclitaxel[2-10], but the levels of production are too low to be useful for commercialization until now. To find endophytic fungi that can produce paclitaxel, we isolated a lot of endophytic fungal strains from *Taxus*. *wallichiana* var. *mairei* (Lemée et H. Lév.) L. K. Fu et Nan Li distributing in Taihang Mountain in Henan province of China. Among these endophytic fungal strains, one strain that can produce paclitaxel was isolated and its production was authenticated with LC-MS. This discovery is very significant for paclitaxel manufacture and environmental protection of yew trees.

Materials and methods

Materials

Instrument: HPLC instrument, Shimadzu GC-2010. LCQ Advantage LC-MS. Lectronic analytic balance (precision: 0.0001). Ultrasonator. Rotary evaporator. Super-clean bench (sterile cabinet). Shake culture box (platform shaker).

Reagents: Methanol (AR). Ethanol (AR). Acetonitrile (HPLC grade). Potato dextrose agar (PDA) culture medium. Potato dextrose broth (PDB) culture medium. paclitaxel (99.5%) was purchased from Sigma Company (St. Louis, MO, USA.).

Materials: The fresh bark was collected from branches of *T. wallichiana* var. *mairei* growing in the Taihang mountain, Henan province, China in March, 2013. The branches were 3-9 cm in diameter.

Methods

Isolation of endophytic fungus

The withered outer layer of fresh bark was removed with a blade and then the bark was surface sterilized with 75% (v/v) ethanol for 2 min, followed by 0.1% mercuric chloride solution for 8 min in the super-clean bench. The surface sterilized bark was washed three times with sterilized water for 1 min each and then cut into pieces (approximately 4 × 4 cm) with the aid of a flame-sterilized blade. Each sterilized piece was placed in a petri dishes (8 cm diameter) containing potato dextrose agar (PDA) culture medium for incubation at 20-22°C for 4-6 days. The three petri dishes containing uninoculated sterile medium were taken as control. When the hyphal tips of endophytic fungi grew out from the bark, they were islolated with a flame-sterilized inoculating blade and sub-cultured on PDA plates to obtain isolated colonies. Each fungal culture was frequently checked for purity.

Screening of endophytic fungi producing paclitaxel

Submerged fermentation of endophytic fungi: Three agar plugs (approximately 4 mm diameter) containing mycelia (same strain) were inoculated into 250 ml culture flask containing 100 ml of potato dextrose broth (PDB) for incubation at 120 rpm and 21-22°C for 4 days. Then these mycelia were subcultured (4-5 ml of fungal liquid culture was inoculated into a 250 ml culture flask containing 100 ml of PDB) for incubation at 120 rpm and 21-22°C for 8 days. The three culture flasks containing uninoculated sterile medium were taken as control.

Preparation of extract: Fungal cultures were filtered with filter paper at first. Then the mycelia and the culture medium were extracted respectively. The mycelia were dried at 45°C, weighed and then were ground in a mortar with quartz sand. The ground mycelia were extracted with 30 ml of chloroform/methanol (1:1, v/v) in an ultrasonic bath for 30 min before filtration. The extraction was repeated (the residue was extracted once again) and the pooled filtrates of two extractions were evaporated under reduced pressure at 40°C in a rotary vacuum evaporator. The residue was dissolved in 30 ml chloroform and then back extracted with 30 ml of water. The organic phase of mixture was collected, evaporated at 40°C in the rotary vacuum evaporator again and then the residue was dissolved in 5 ml methanol and filtered with 0.45 μm filter. This filtrate was referred to as mycelial extract. Filtered spent culture medium was evaporated under reduced pressure at 70°C in the rotary vacuum evaporator and then was extracted with the same method described for ground mycelia. The last filtrate of culture medium was referred to as spent culture medium extract. The culture from another culture flask was filtered and then the spent culture medium was evaporated under reduced pressure at 70°C in the rotary vacuum evaporator. The residue was mixed with dried ground mycelia of the same culture flask and the mixture was extracted using the same method described for ground mycelia. This filtrate of culture was referred to as whole culture extract.

Determination of paclitaxel

The HPLC column used in determination of paclitaxel was a Shimadzu C_{18} reverse phase column (5 μm, 250mm×4.6mm). The volume of extract injected was 10 μl. The gradient mobile phase consists of acetonitrile and water. The content of acetonitrile in the gradient mobile phase varies as below (v/v): from 27% to 30%

in 0-15min, 30% to 37% in 15-30min, 37% to 42% in 30-40min, 42% to 47% in 40-60min and 47% to 48% in 60-72min. The flow rate was set as 0.8 ml/min. The column temperature was 35°C. A variable wavelength recorder set at 228 nm was used to detect ingredients eluted from the column. Standard paclitaxel solutions were prepared at 0.0005, 0.001, 0.004, 0.01, 0.02 and 0.05 mg/ml respectively and analyzed according to the above HPLC method. The chromatography peak areas of paclitaxel were recorded to prepare a standard curve (relating peak area to its content) with SPSS (Statistical Product and Service Solutions). Fungal paclitaxel in the extracts was determined with the same HPLC method described above. The content of fungal paclitaxel in extracts was analysed according to chromatography peak areas and the standard curve.

Spectroscopic analysis of extracts

The fungal paclitaxel sample was collected from HPLC column during the retention time (begin at 67.7 min and end at 68.7 min). Electrospray ionization-tandem mass spectrometry (ESI-MS/MS) analysis was conducted on fungal paclitaxel sample. ESI was used as the ion source, with scanning in the negative ion mode. The range of molecular weights scanned was set at 50-2000 amu. The sample was injected with 0.2 μl/min spray flow using N_2 as atomization gas and auxiliary gas. The spray voltage was set at 5.0 kV. The temperature of the capillary cone was set at 350°C.

Identification of endophytic fungi

The paclitaxel producing endophytic fungus was identified by Taihegene Biotechnology Co Ltd with 26S rDNA D1/D2 sequence PCR and ITS Sequence PCR.

Results

A total of 435 endophytic fungal strains were isolated from *T. wallichiana var. mairei* and purified. The retention time of a peak in the chromatograms of mycelial extract, spent culture medium extract and culture extract prepared from isolate No. 53 was identical with that of standard paclitaxel (Fig. 1).

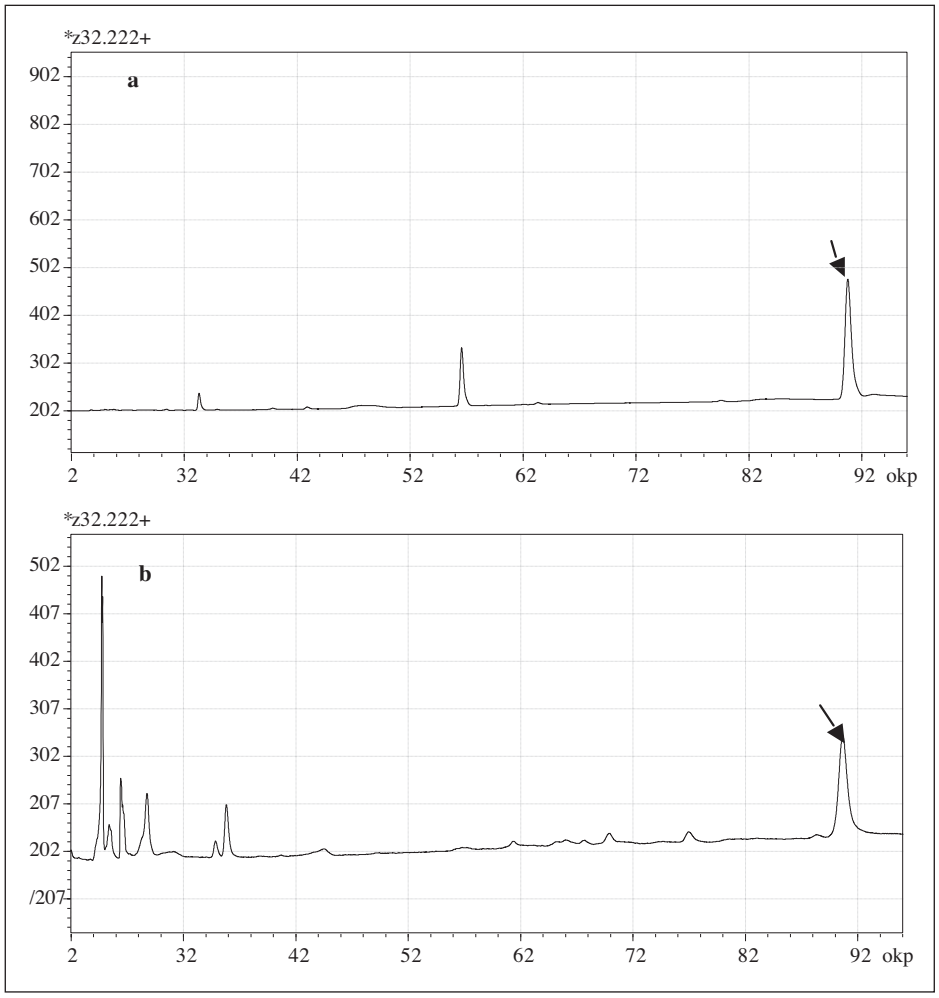

Fig. 1.—Chromatogram of standard (a) and paclitaxel in extraction (b).

No peak was found at that retention time in the chromatograms of extracts from any of the other strains, or from any blank culture samples. The presumptive paclitaxel sample prepared from fungal isolate no. 53 was analyzed with Electrospray ionization-tandem mass spectrometry (ESI-MS/MS). There are molecular or ions in the Mass spectrum of the fungal paclitaxel sample, which possesses same molecular weight (or W+H) as that of moleculars or ions in the Mass spectrum of paclitaxel standard. The mass spectrometry analysis of the fungal paclitaxel sample confirmed that there is paclitaxel in the culture of isolate no. 53 (Fig. 2).

The paclitaxel concentrations detected in extracts of the whole culture, the mycelium and the spent culture medium from isolate no. 53 were 0.18 mg/L, 0.041mg/L and 0.143 mg/L respectively according to the standard curve (Table I).

Therefore, the paclitaxel content in whole PDB culture and that in spent culture medium from isolate no. 53 is 0.0153 mg/L and 0.0119 mg/L respectively. The paclitaxel content in dry mycelium is 0.27 mg/kg.

The similarity between 26S D1/D2 rDNA sequence (accession numbers: KP939361) of isolate no. 53 and that of *Fusarium* was higher than that between it and any other genus, at 99%. The similarity between the ITS1/ITS2 rDNA sequence (accession numbers: KP939362) of isolate no. 53 and that of *Fusarium SP*. was also higher than that between it and any other species, at 99%. Therefore, isolate no. 53 belongs to *Fusarium*. But there is not unique species of which the 26S D1/D2 rDNA sequence or ITS1/ITS2 rDNA sequence match with that of isolate no. 53. This strain is probably a new species. This strain grew well on PDA at 20-22 °C with thick white hypha. The strain can secrete red substance that permeate into medium.

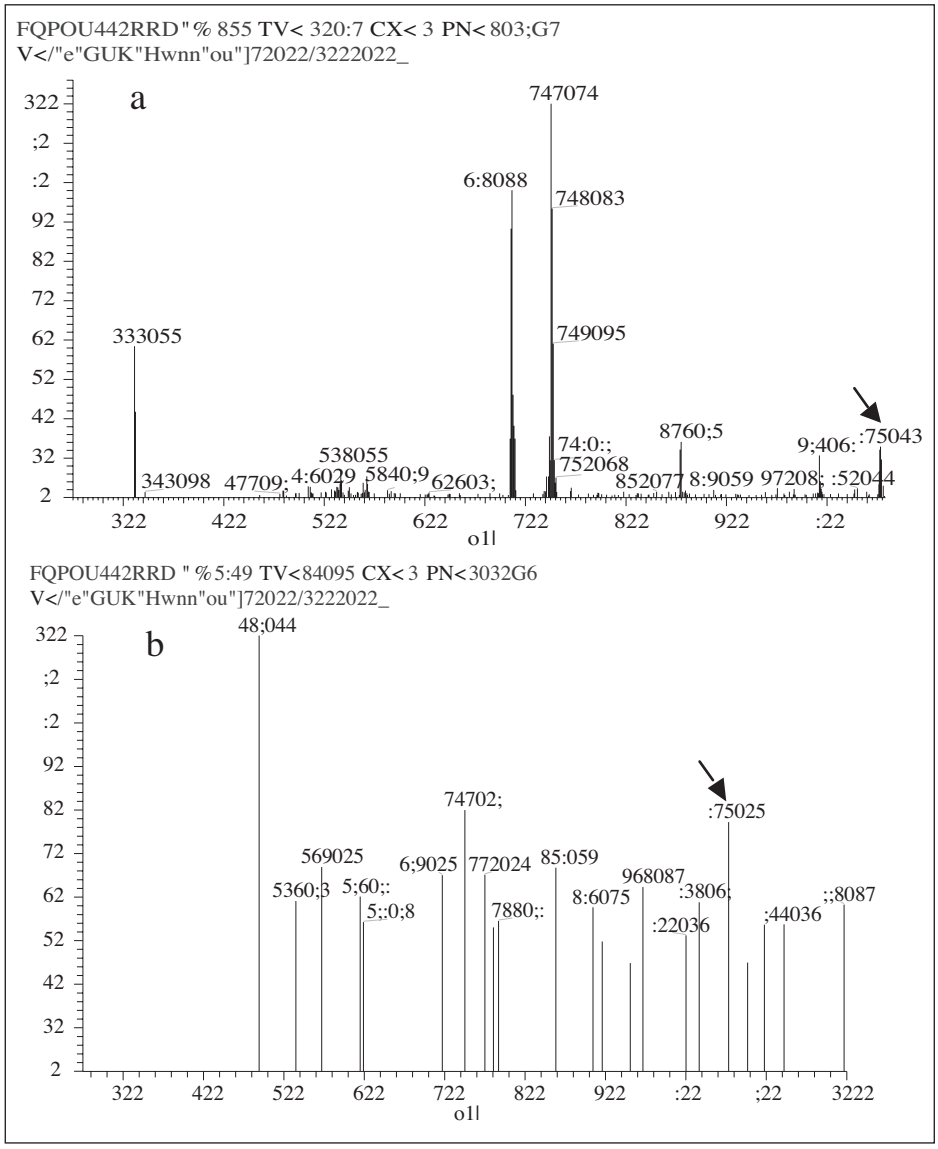

Fig. 2.—MS spectrums of standard (a) and paclitaxel in extraction (b).

Table I
Standard curve and the contents of paclitaxel in extracts and culture

Sample	*Retention time (min)*	*Content in extract(mg/L)*	*Content in culture(mg/L)*
Standard	68.167	$C=4A\times10^{-8}-0.000587$ (C: content in extract. A: peak area) $R^2=0.9999$	
Whole culture	68.135	0.18	0.0153
Spent culture medium	68.213	0.143	0.0119
Mycelia	68.182	0.041	0.27 mg/kg

Discussion

The isolation of an endophytic fungus producing paclitaxel is very significant to paclitaxel manufacture and resource protection of taxus. Until now, researchers have found more than 100 species in more than 20 genera endophytic fungi that produce taxol, such as *Axomyces andreanae*, *Estalotiopsis microspora*, *Alternaria alternata*, *Periconia* sp., *Pithomyces* sp., *Chaetomella raphigera*, *Monochaetia* sp. and *Seimatoantlerium nepalense*[1,11-12]. Although there are much of reports on the isolation of endophytic fungi producing paclitaxel from yew, but all of the contents of paclitaxel in the reported isolates were low for immediate commercialization[1]. The endophytic fungus producing paclitaxel reporteded in our study was isolated from *T. wallichiana* var. *mairei* in the Taihang Mountains in Henan province of China. The fungal paclitaxel was determined and authenticated with LC-MS. Although the paclitaxel yield of this endophytic fungus strain is low, but this strain enlarges the family of endophytic fungi producing and shows potentiality as a strain producing paclitaxel for manufacture after strain improvement. Our strain producing paclitaxel was identified belong to *Fusarium* from its ITS rDNA and 26S D1/D2 rDNA sequence. There is not unique species of which the 26S D1/D2 rDNA sequence or ITS1/ITS2 rDNA sequence match with that of our isolate, which indicate that this strain is probably a new species or variety and need consequent study. There are several strains belong to *Fusarium* that could produce paclitaxel have been reported previously[11,13-18]. Even a strain of *Fusarium solani* can produce baccatin III (paclitaxel precursor)[19]. These show that *Fusarium* is a very valuable genera possesses the gene system of paclitaxel and can be taken original strain to breed paclitaxel manufacture strain.

Although there are lots of endophytic fungi in yew, But seldom strain can produce paclitaxel. In the 435 endophytic fungal strains isolated in our study, we found only one strain that could produce paclitaxel. In this study, we determined the contents of paclitaxel in a mixture of mycelia and spent culture medium, in the mycelia alone and in spent culture medium alone. In other studieds, only the content of paclitaxel in mixtures of mycelia and spent culture medium were reported[7-8,20-22]. In our study, the content of paclitaxel in spent culture medium is close to that in whole PDB culture, which indicating that most paclitaxel synthesized is secreted into the culture medium. This isolated endophytic fungus produced paclitaxel at a considerable level and shows potentiality as a producing strain for paclitaxel manufacture after strain improvement.

Interests Declaration

The authors of this article declare that they have no conflicts of interests.

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