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EXPERIMENTAL ARTICLES

Isolation and Fatty Acid Analysis of Lipid-Producing Endophytic Fungi from Wild Chinese *Torreya Grandis*[1]

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Abstract—*Torreya grandis* is a rare tree species of economic value. Its seeds are rich in lipids, although their use is limited by their high cost. Recent studies demonstrated that certain endophytic fungi can produce metabolites similar to those produced by the host plant. In the present study, endophytic fungal strains capable of producing lipids were isolated from wild Chinese Torreya grandis. Among these, the XF-38 strain had the highest lipid content at 17.68%. Comparative analysis of the fatty acid composition of lipids produced by endophytic fungi and *Torreya* seeds by GC-MS showed that the two sources shared some of the same components, including linoleic acid, oleic acid, and sciadonic acid (5c, 11c, 14c-eicosatrienoic acid). The morphology and internal transcribed spacer rDNA of the XF-38 strain were determined, and a BLAST search showed that this strain belongs to *Bionectria ochroleuca*. Finally, generation of a growth curve and determination of the lipid content of strain XF-38 confirmed that lipid production was related to the amount of mycelium, and the lipid content resched 3.1 g/L in the microcrystalline cellulose meduim.

Keywords: *Torreya grandis*, endophytic fungi, fatty acid, isolation, sciadonic acid

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INTRODUCTION

The deterioration of the Earth's environment could lead to the extinction of an increasing number of ancient trees, such as *Torreya grandis*, which is a tree species native to China that belongs to the *Taxaceae Torreya* genus of evergreen trees. This expensive tree can live for up to 400–500 years, and is therefore known as the longevity tree, and research on its content of physiological active substances only began in recent years (Chen et al., 2006; Muhammad et al., 2010). *Torreya grandis* seeds can be used medicinally for the treatment of productive cough, thirst, hemorrhoids and to expel parasites from the intestinal tract, among other conditions. The seeds have a high lipid content with a high proportion of unsaturated fatty acids, including rare fatty acids such as sciadonic acid (Wolff et al. 1999; Dai et al., 2007). However, *Torreya* seeds require 3 years from blossoming to maturation, and they are scarce and expensive. It is difficult to obtain of the special nutrients in seeds of *Torreya grandis* by traditional methods.

Endophytic fungi has become a new material that can produce some food effective components in recent years. It grows in healthy living plant tissues without causing disease symptoms in the host. Their presence is not associated with any symptoms in the host. The long-term coexistence of endophytic fungi with their plant hosts suggests a period of co-evolution during which the exchange of genetic information, may have resulted in the production of the same or similar biologically active components by the two organisms (Stierle et al., 1993; Gary et al., 2006), such as amide, cyclohexanone (Vatcharin et al., 2013), and mycodiesel hydrocarbons (Gary at al., 2008). Recent studies found that endophytic fungi isolated from oil-seed crops, particularly woody oil crops, can produce a variety of fatty acids. A new unsaturated fatty acid, (4E)-6,7,9-trihydroxydec-4-enoic acid, was identified in the endophytic fungal strain phomopsis sp. NXZ-05 of *Camptotheca acuminate* (Tan et al., 2007). Endophytic fungi isolated from the Chilean gymnosperm *Prumnopitys andina* can produce fatty acids (Schmeda et al., 2005), and those colonizing Atractylodes lancea are capable of producing essential fatty acids (Teng et al., 2013). An endophytic fungus from Aloe arborescens produces short-branched fatty acid dimers (Teigo Asai et al., 2013).

The study of *Torreya grandis* endophytic fungi has gradually expanded since Stierle et al. showed that endophytic fungi can produce the same metabolites as their host plants in 1993. However, most studies performed to date have focused on the identification of antibacterial substances (Huang et al., 2001), whereas research on fatty acids is limited. It is a great limitation

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for large-scale application of special lipids of *Torreya grandis* seeds.

In this study, the fatty acid composition of lipids produced by endophytic fungi isolated from wild Chinese *Torreya grandis*, was compared to that of lipids originated from their host plant. It is expected that the microorganism fermentation techniques could be used for the production of lipids of a specific composition to substitute those of *Torreya grandis* seeds, which could be of value for the manufacture of foods. This could serve not only to promote the protection of rare species, but also to reduce production costs and improve the application value.

MATERIALS AND METHODS

Plant Material

Fresh *Torreya grandis* tissue samples were collected from the Zhong Jia Ling *Torreya grandis* Park, Zhejiang Province, China. The park has the largest area planted with this tree in the world, and includes more than three lines of wild Chinese *Torreya grandis* old trees.

Medium and Staining Solution

Water agar (WA) medium and potato dextrose agar (PDA) medium were used to isolate and purify endophytic fungi. Potato dextrose broth (PDB) medium was used for the fermentation of endophytic fungi. Sudan black dye (Sudan black B 0.3 g, dissolved in 100 mL of a 70% ethanol solution, mixed by forced oscillation) was used for fatty acid production.

Isolation of Endophytic Fungi

Plant tissue samples were washed and dried, and the central stem was cut into 0.5 cm pieces, which were placed in a 70% ethanol solution for 60 s and treated with a 3.5% sodium hypochlorite solution for 3–6 min. The mixture was then placed in 70% ethanol for 30 s and washed three times with sterile water. The stem segments were incubated in WA at 25°C and cultured in the dark for 7 days for colony formation.

Liquid Culture and Fermentation Processing of Endophytic Fungi

The mycelium or spores of endophytic fungi were inoculated into PDB liquid medium with shaking at 120 rpm at 25°C in the dark until the appearance of a large number of mycelia. The fungus was then inoculated into fresh PDB medium(the volume of flask/medium is 250/50 mL) at 10% on a shaker at 120 rpm and 25°C for 7 days. The mycelia were then collected and freeze-dried.

Screening of Lipids Produced by the Endophytic Fungi

The freeze-dried mycelia was added to Sultan black dye (1–2 mL) at room temperature for 5 min. The dyed mycelia were washed in water for 5 min and naturally dried. The lipid droplets in the dyed mycelia were observed by microscope.

Determination of Lipid Content

Strains were cultured in PDB liquid medium at 25°C for 6 days on a shaker at 120 rpm and collected after cleaning. Samples were put in a test tube with 1 g culture, added to 6 mL HCl (4 mol/L) and placed in a boiling water bath for 30 min. Then, a volume of chloroform methanol mixed solvent (V/V = 2/1) equal to twice the volume of the sample was added and quick-cooled at –20°C for 3 min. The chloroform layer was collected after centrifugation at 5000 rpm for 5 min. Finally, 0.1% NaCl solution was added, the mixture was centrifuged at 5000 rpm for 5 min and the chloroform layer was collected and dried at 50°C. The liquid quality after drying reflects the oil quality. Quality/fat = the fat cell dry weight (1 g) × 100%.

Analysis of the Fatty Acid Composition of Endophytic Fungi

For methyl ester analysis, 1 mg of sample was added to 2 mL of 0.6 mol/L KOH-methanol and 2 mL hexane and after 2 min mixing, 5 mL water was added for 15 min at 30°C. The extract was analyzed by gas chromatography-mass spectrometry (GC-MS) (U.S.A., Agilent 6890N/5973). The sample was incubated at 50°C for 2 min, then the temperature was raised to 280°C by a 15°C/min gradient, and the sample was maintained at this temperature for 20 min.

Chromatographic conditions: the chromatographic column used was Rtx25MS (300 mm × 0.25 mm × 0.25 μm) with helium as a carrier gas and a flow rate of 1.63 mL/min. The column temperature was 80°C (maintained for 3 min). The sample was heated to 130°C (maintained for 5 min) with an inlet temperature of 30°C, an injection volume of 1 μL, and a split ratio of 30:1.

MS: the ionization source EI had an electron energy of 70 eV; the ion source temperature was 200°C, with a detecting temperature of 250°C, solvent delay of 3 min, scanning range of 30–550 amu, and a doubling voltage of 1.2 kV. The retrieval database was identified by the NIST mass spectra, and was quantified by the peak area normalization method.

Identification of Endophytic Fungi

The internal transcribed spacer (ITS) sequence was analyzed using the Chromas Genetyx software and homology analysis was performed in the NCBI gene pool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was made by MEGA5.1. The SK1375 fungal genomic DNA extraction kit was used and primer sequences were as follows: ITS1, 5' TCCGTAGGTGAACCTGCGG 3'; ITS4, 5' TCCTCCGCTTATTGATATGC 3' (Guo et al., 2001). PCR conditions were as follows (50 μL total volume): template,

Table 1. Plant material and lipid content of endophytic fungi

No.	Strain	*Torreya grandis* type	Tree-age, year	Lipid, %
1	XF-4	No grafting	≈50	6.37
2	XF-21	Great circle *Torreya* (mail)	≈100	8.41
3	XF-36	Sesame-*Torreya* (femail)	>500	15.33
4	**XF-38**	**Sesame-*Torreya* (femail)**	**>500**	**17.68**
5	XF-59	Sesame-*Torreya* (femail)	≈50	9.56
6	XF-72	No grafting	≈40	6.98
7	XF-75	No grafting	≈40	6.01
8	XF-81	Thin-*Torreya* (mail)	≈70	7.21
9	XF-89	Thin-*Torreya* (femail)	≈70	8.78
10	XF-95	Thin-*Torreya* (femail)	≈60	7.91

Table 2. The GC-MS diagram of fatty acid of *Torreya grandis* seed and XF-38

No.	Fatty acid	Relative mass fraction, %	
		Torreya seedXs	XF-38
1	Myristic acid	/	1.37
2	Pentadecanoic acid	/	1.17
3	Palmitic acid	8.22	7.38
4	Hexadecenoic acid	/	3
5	Stearic acid	4.48	3.46
6	Oleic acid	38.36	40.23
7	Linoleic acid	32.15	23.76
8	Linolenic acid	0.54	0.64
9	Benzoic acid	/	0.35
10	Arachidic acid	0.41	0.36
11	Eicosenoic acid	1.05	1.19
12	Eicosadienoic acid	1.89	1.79
13	Sciadonic acid	6.69	7.11
14	Docosanoic acid	7.97	8.19

10 pmoL; primer up (10 μM), 1 μL; primer down (10 μM), 1 μL; dNTP mix (10 Mm each), 1 μL; 10× Taq reaction buffer, 5 μL; Taq (5 μg/μL), 0.25 μL; water added to 50 μL. Pre degeneration was performed at 98°C for 5 min, followed by 95°C for 35 s, 55°C for 35 s, 72°C for 40 s, 35 cycles, and an 8 min final extension.

Generation of Growth Curves

The strain was inoculated into PDB medium at 25°C with agitation at 120 rpm. Fermentation liquid was removed at specific times and centrifuged for 15 min at 5000 rpm. Because lipid production was mainly related to the carbon source, glucose were replaced by 2% microcrystalline cellulose in PDB. The mycelia were collected and freeze dried. The dry weight and fat content of the mycelia were measured and growth curves were generated.

RESULTS AND DISCUSSION

Isolation of Lipid-producing Endophytic Fungi from Wild Chinese Torreya grandis

Native male and female *Torreya grandis* tree species of different ages (30–500 years) were used, and 106 strains of endophytic fungi were isolated and numbered from XF-1 to XF-106. The 106 strains isolated were subjected to Sultan black staining, which resulted in the identification of 47 strains of mycelia with small black spots containing large oil drops. The results of ten strains whose have highest lipids showed in Table 1. Lipid-producing endophytic fungi were isolated from female and native *Torreya* trees with no grafting, and production levels were positively correlated with age. Endophytic fungi of an advanced age produced a greater amount of lipid. Among the strains examined,

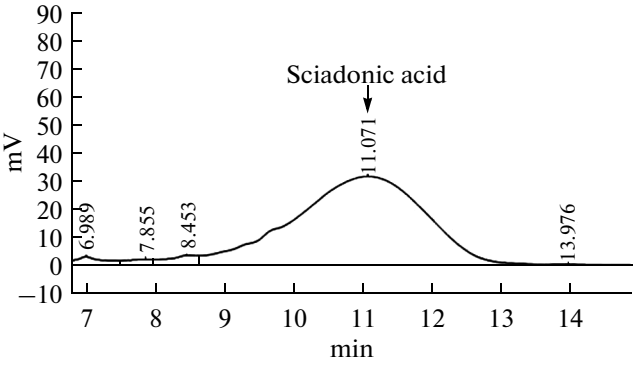

Fig. 1. The GC-MS diagram of fatty acid of sciadonic acid of XF-38.

the XF-38 strain, which was isolated from a 500-year-old over female Torreya grandis tree, produced the greatest amount of lipid. In this strain, the lipid content reached 17.68%, which was the highest of all strains.

Fatty Acid Composition of XF-38 Strain and Torreya grandis Seeds

The fatty acid profile of the XF-38 strain and *Torreya grandis* seeds are showed in Table 2. The results showed that the XF-38 strain could produce 14 types of fatty acids. Among them, myristic acid, pentadecanoic acid, hexadecenoic acid and benzoic acid were only produced by endophytic fungi, whereas the remaining 10 fatty acids were produced by both *Torreya grandis* seeds and endophytic fungi, and both had a similar relative content except for the amount of linoleic acid. We speculated that this could be caused by the inhibition of a ω^{12}-fatty acid dehydrogenase, or that the metabolic pathway for the production of fatty acids in strain XF-38 is more complex than that of *Torreya grandis*. In addition, sciadonic acid (5c, 11c, 14c-eicosatrienoic acid), which is a characteristic fatty acid of pine, ginkgo, and other gymnosperms, was identified as a metabolite produced by strain XF-38. Sciadonic acid has obvious antibacterial and anti-inflammatory effects (Tanaka et al., 2001; Berger et al., 2002), low triglyceride levels, and it possesses specific physical properties (Endo et al., 2006) in addition to being a rare species of ω-6 polyunsaturated fatty acid. Until now, there hasn't relevant report about sciadonic acid producing endophytic fungi. The part of sciadonic acid is shown in Fig. 1.

Identification of Strain XF-38

The mycelium of XF-38 produced abundant microconidia that appeared cylindrical to oval, creamy white, and without vibration. Macroconidia were formed 4–6 days after inoculation on the PDA medium and formed a pellet of 2–4 mm by liquid fermentation, the pictures are shown in Fig. 2. The

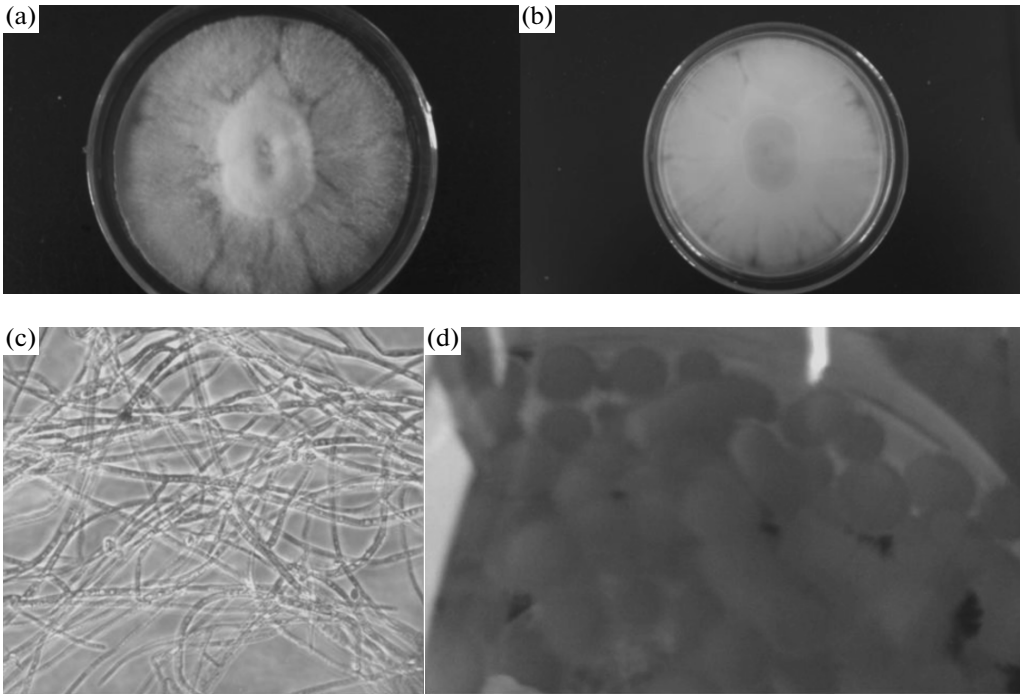

Fig. 2. Morphology characteristics of XF-38. (a) Colonies morphology(positive) in PDA medium. (b) Colonies morphology(opposite) in PDA medium. (c) Aerialhyphae using a microscope (400 times). (d) Mycelium pellet formation in PDB medium.

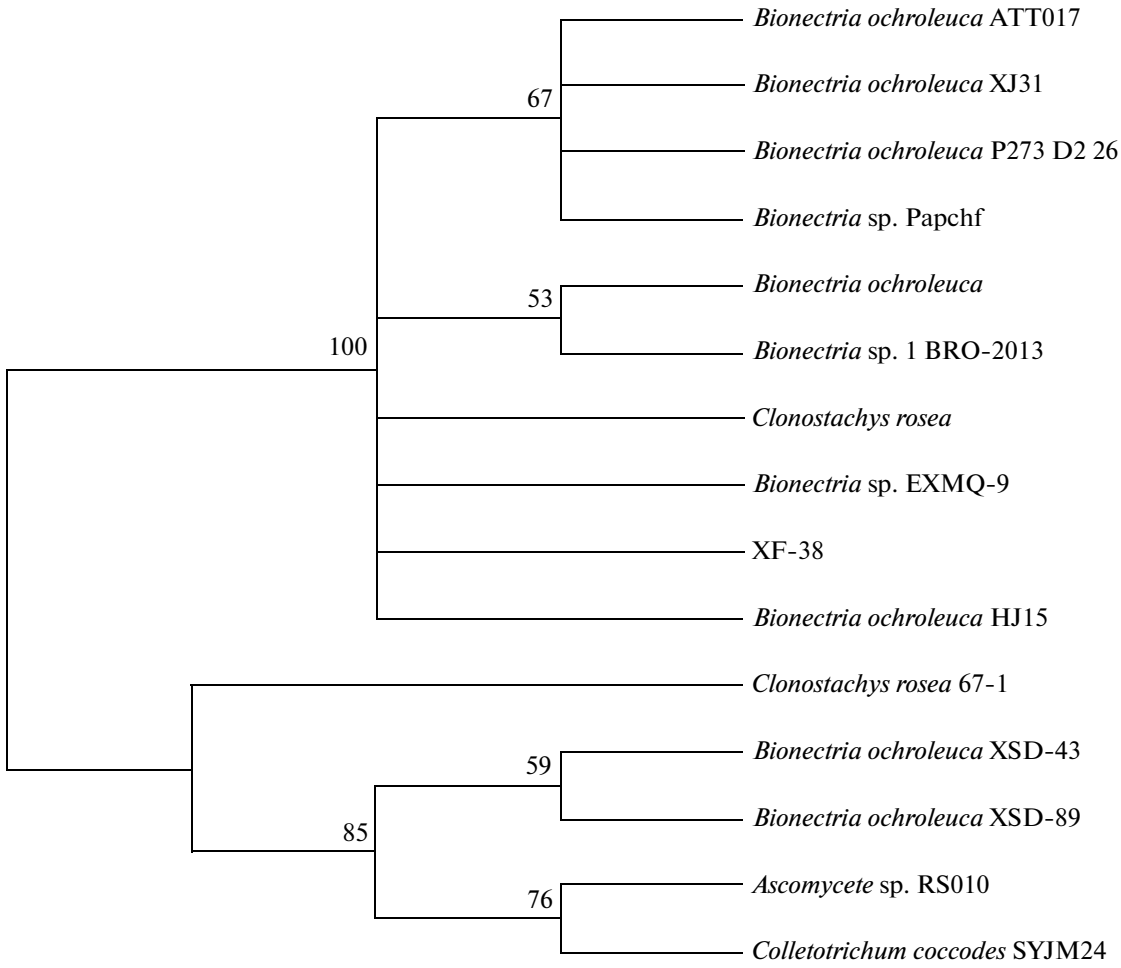

Fig. 3. Phylogenetic tree of XF-38.

sequence was obtained from the NCBI GenBank database and the ITS rDNA sequence of strain XF-38. A BLAST search showed that the isolated strain XF-38 had 100% sequence homology with *Bionectria ochroleuca*, as seen by a 99% query coverage. The result of phylogenetic tree showed in Fig. 3.

Bionectria ochroleuca was independent from *Nectria sinensis* in 1999, its anamorph is *Clonostachys rosea* (Schroers et al., 1999). There had a report about lipid producing *Bionectria* strain, which was isolated from *Pinus massoniana*, but the fatty acid content and types were not clear(Deng et al., 2014). In addition, the research about *Bionectria ochroleuca* mainly focused on the antibacterial activity, such as paclitaxel (Yu et al., 2007), TMC-151 A Monoacetate (Yu et al., 2007), and so on. Endophytic fungi isolated this study could produce special fatty acid such as sciadonic acid, which can provide good material for the study of acid synthesis enzyme. The future study about XF-38 may be can start from the physiological active substances.

Determination of the Growth Curve

The mycelium weight and the fat content resulting from fermentation are shown in Fig. 4. (a) showed that at the 72 h time point, the mycelium and oil content had increased, and the volume remained stable from 96 to 168 h, when the mycelial biomass decreased slightly in PDB. The liquid culture time ranged from 96 to 144 h, and consistent with the results of screening, the quality of the mycelium and the lipid content, which remained at approximately 18%, were relatively stable. (b) showed that XF-38 could grow in medium used microcrystalline cellulose as carbon source, the growth curve and lipid content were similar in PDB before the 96 h time point, and the lipid content reached 3.1 g/L. It suggested that the ability to produce lipids is relatively stable for XF-38.

At present, there are many researches about lipid-producing microbe, the utilization of cellulose is important condition for application, and XF-38 strain had satisfied it. Carbon source were used the cellulose

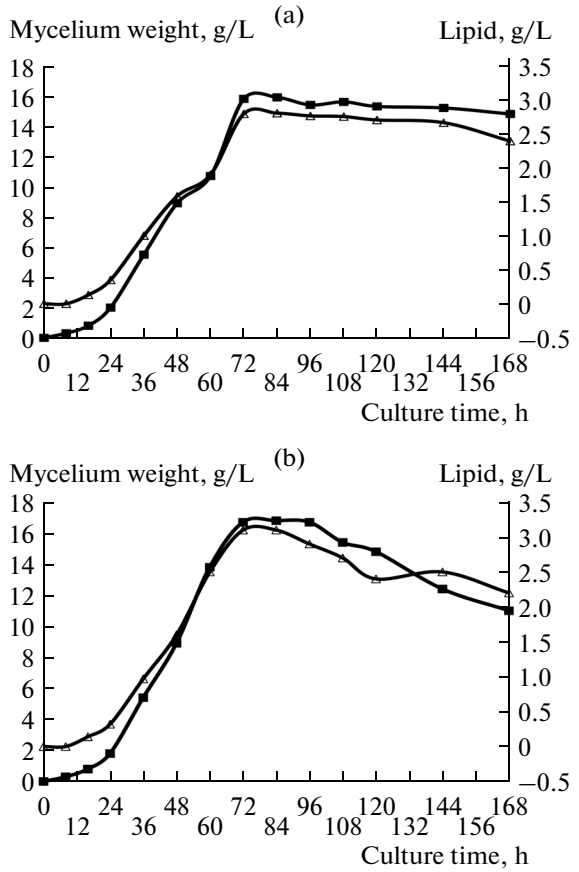

Fig. 4. Growth curve of XF-38 and production of lipid content ■ mycelium weight, △ lipid content. (a) Glucose as carbon source, (b) microcrystalline cellulose as carbon source.

instead of glucose, it can not only reduce the production cost, but also expand the material resources. Move over, study on enzyme for cellulose degrading can be developed for XF-38.

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