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IDENTIFICATION OF FLAVONOIDS AND FLAVONOID-PRODUCING ENDOPHYTIC FUNGI ISOLATED FROM *OPISTHOPAPPUS TAIHANGENSIS* (LING) C. SHIH

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Abstract

Work on endophytic fungi from *Opisthopappus taihangensis* (Ling) C. Shih that produce the major medicinal ingredient, flavonoid was carried out. A total of 37 isolates of endophytic fungi were separated and purified from which 8 flavonoid-producing isolates were selected. Among the 8 isolates, 3 (T9, S9, S10) produced only rutin, 1 (T2) generated only quercetin, 1 (R2) generated rutin and quercetin, and the remaining 3 (T1, T8, R1) simultaneously produced rutin, quercetin, and isorhamnetin. None produced Kaempferol. In addition, highest concentration (mg/ml) of rutin, isorhamnetin and quercetin were produced by R1 (0.0821), S10 (0.046) and S9 (0.0116), respectively. By morphological assessment and molecular (ITS region) identification, eight endophytic flavonoid-producing fungi were belonged to the genera *Fusarium* (2), *Alternaria* (2), *Aspergillus*, *Colletotrichum, Pestalotiopsis* and *Penicillium*.

Introduction

Opisthopappus taihangensis (Ling) C. Shih (Asteraceae) is a perennial herbaceous plant only exists in the Taihang Mountain area of Henan, Shanxi, and Hebei provinces in China. This plant species grows along hillsides and in crevices of cliffs. In addition, because of the recent recognition of its high ornamental and medicinal values, it has undergone severe harvesting, thereby resulting as an endangered plant species of Henan province. Flavonoids are the main medicinal ingredients in *O. taihangensis* and are mainly used for its anti-oxidative, anti-tumor, and cardioprotective activities (Zhang *et al*. 2014).

Endophytic fungi live within the tissues of the majority of plants for, at least, part of their life cycles, without showing any obvious disease symptoms. Endophytic fungi produce secondary metabolites that are utilized by the host plant to improve its resistance to stress (Campos *et al*. 2015), as well as to generate bioactive compounds, such as antibiotics, enzyme inhibitors (Combes *et al*. 2012), flavonoids, and acetylene derivatives (Bezerra *et al*. 2015). Previous studies have shown that endophytic fungi produce flavonoid compounds similar to those generated by the host plant. Current studies have focused on antibacterial (Dos *et al*. 2015) and anticancer drug (Tao *et al*. 2015) development using extracts from endophytic fungi. The identity of its endophytic fungi, as well as the bioactive compounds, including flavonoids produce by the fungi remain unclear. In the present study, flavonoids and flavonoid-producing endophytic fungi were identified on the basis of their color reactions, examined by high-performance liquid chromatography (HPLC), and morphological and molecular characteristics, respectively.

Materials and Methods

Wild *Opisthopappus taihangensis* plants were collected from the Jiyuan Taihang Mountain and planted in experimental plot. Healthy new and old leaves were randomly selected and after washing in running tap water and air-drying, the leaves were stored in a refrigerator at 4ºC up to 48 hrs.

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Potato dextrose agar (PDA), BEPA (Chen *et al*. 2016), Czapek's (Pohjanen *et al*. 2014), and Gause's synthetic agar (Gonzalez-Teuber *et al*. 2014) were used for isolation of endophytic fungi following Agar Plate method. PD broth was used for shake flask fermentation culture. Surface sterilization of inocula consisted of the following steps: first, leaves were soaked in the detergent solution containing sodium alkylsulfonate 2% for 30 min. After soaking, the detergent was washed out in tap water for 30 min. The leaves were air-dried on a laboratory desk. On the super clean bench, the leaves were washed with sterile water thrice, immersed in 75% ethanol for 45 sec, washed again with sterile water thrice, soaked in 0.1% $HgCl_2$ for 45 sec, washed with sterile water 3 - 5 times, and finally blotted with sterile filter paper. Two types of leaves above were cut into about 2 mm × 2 mm small pieces using sterilized scissors. The leaf pieces were then placed on the endophytic fungi separation medium which were sterilized, with about 20 pieces in each medium tablet. Once hypha branched out from the leaf blade incision, these were collected from the top of the hypha. Each colony was purified by picking and inoculating onto a new tablet 3 - 4 times. Images of single colonies and microphotographs of each isolate were captured, and their growth was recorded for subsequent analysis. The sterile water used in the last rinse was set aside, and was used following spread plate method to test the effectiveness of surface sterilization of the inocula and exclusion of non-endophytic fungi from the inocula.

Cultures were grown at room temperature or at 28°C in a constant temperature incubator and were kept in the dark for 3 to 5 days. The fungi isolates cultured in liquid PDA medium were kept on a shaking table for 5 - 7 days at 28°C. All culture media were sterilized prior to inoculation. Isolated endophytic fungi were preserved in sterile water (Lalaymia *et al*. 2012). To be specific, 1 ml sterile water was injected into a 1.5 ml sterile centrifuge tube, and 0.5 cm × 0.5 cm agar block with hypha was stored in the sterile water at 4°C.

Solution (w/v) of quercetin (0.25%), rutin (0.30%), kaempferol (0.20%), and isorhamnetin (0.35%) were used as flavonoid standard samples for HPLC (Costa *et al*. 2014). The standard samples were then, respectively diluted to 0.2, 0.4, 0.6, 0.8 and one time the initial concentration (Yam *et al*. 2010). Prior to HPLC testing, all samples were filtered through a 0.45 μm membrane filter. The HPLC testing conditions were as follows Chromatographic column: C_{18} (150 mm × 4.6 mm, 5 μm); column temperature: 28°C; mobile phase $V_{methanol}$: $V_{0.3\ \%\ phosphoric\ acid}$ = 55 : 45; flow rate: 0.8 ml/min; detection wavelength: 370 nm; and sample volume: 8 μl (Obmann *et al*. 2012). The fermented product was subjected to ultrasonication for 1 hr (Peter *et al*. 2015), filtered by using a vacuum suction and sterile filter paper, a 100 ml aliquot of the filtrate was concentrated at 45°C in vacuum conditions, and then diluted with water to a final volume of 5 ml. Prior to HPLC analysis, the samples were divided into vials with a 1 ml, centrifuged at 12,000 rpm for 10 min and then filtered through a 0.45 μm membrane filter.

Present authors screened flavonoid-producing endophytic fungi by using two methods, namely the flavonoid color reaction of fermented liquid and the color reaction of mycelial isolates. They inoculated one ring of the stored isolates into PDA liquid medium, cultured them with constant shaking at 120 rpm for 3 - 5 days at 28°C. Then, the cultures were centrifuged at 3,500 rpm for 15 min to collect the fermented liquid and mycelia. Sterile filter paper was used to suck out the water from the mycelia, which were dried at 30°C for 6 hrs and then stored at 4°C. These flavonoid-producing isolates were then tested for flavonoid production using a colorimetric reaction. The dry mycelia were ground into powder and soaked overnight in a leaching solution (7 : 3 methanol : concentrated hydrochloric acid) at a solid:liquid ratio of 1 : 20 (Wang *et al*. 2016). After this pretreatment, the mixture was subjected to ultrasonication for 1 hr (power: 35%, pulse: 3 sec, interval : 3 sec) (Yoo *et al*. 2012). Finally, the mixture was centrifuged at 12,000 rpm for 10 min, after which the supernatant fluid was collected, dried under a vacuum in a 45°C environment, and then diluted with water to a final volume of 10 ml. In the experiments, each liquid sample that

was poured drop-wise onto a filter paper until dry, subsequently, a chromogenic agent was dropped at the sample point. The resulting color reactions were then used to identify each specific flavonoid following Table 1.

Table 1. Five kinds of colorimetric reactions for flavonoids detection (Yu *et al.* 2004).

Developer	Test conditions	Positive result	Resulting flavonoid
$AlCl_3$	365 nm UV-light	Tawny fluorescence	Flavonoids have 3-hydroxy, 4-carbonyl or 5-hydroxy, 4-carbonyl or catechol hydroxyl
Al $(NO_3)_3$-$NaNO_2$	UV-light, observed immediately	Red fluorescence	Flavonoids have 3-hydroxy, 4-carbonyl or 5-hydroxy, 4-carbonyl or catechol hydroxyl
NaOH	Overnight reaction	Yellow	Flavone, chalcone, aurone, flavonol
HCl-Mg	Boiling water bath	Orange-red to red-violet	Flavone, flavonol, flavanone, flavanonol
$FeCl_3$	Overnight reaction	Blue	May have a phenolic hydroxyl group

The flavonoid-producing endophytic fungi isolated in the present study were identified through traditional morphological and molecular assessment. Traditional morphological identification involved the observation of colony and spore morphology and microscopic determination of intrinsic characteristics.

Genomic DNA of endophytic fungi was extracted using a Genview fungus genomic DNA extraction kit (Beijing, China), and the universal primers ITS1 (5'-TCCGTAGGTG AACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') were employed to amplify the fungal ribosome rDNA ITS sequence. After BLAST analyses of the fungal sequences in GenBank, an evolutionary tree was constructed, and combined with the morphological identification results, to classify the flavonoid-producing isolates. Each PCR reaction system contained a total volume of 25 µl, which consisted of the following: 1 µl of the DNA template, 1 µl of the ITS1primer (10 µmol/l), 1 µl of the ITS4 primer (10 µmol/l), 12.5 µl of the mixcontainning DNA polymerase, PCR buffer and dNTP, and 9.5 µl of deionized water. The amplification conditions were as follows: initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 sec, annealing at 58°C for 40 sec, extension at 72°C for 2 min, final extensionat 72°C for 10 min, all of them were performed for a total of 35 cycles. The PCR products were resolved using a 1% agarose electrophoresis gel. DNA marker was employed to estimate the molecular weight of the PCR products. The qualified PCR products were sequenced at the Shanghai Biological Engineering Company.

Results and Discussion

Based on colony morphology, exudates, and microscopic observations of the endophytic fungi, a total of 37 isolates were obtained from the leaves of *Opisthopappus taihangensis,* 29 isolates were isolated in PDA medium, 5 isolates in beef extract peptone medium, 2 isolates in Chavez medium, and 1 isolate in Gause's synthetic agar medium. Twenty eight isolates were obtained from old leaves and 9 were isolated from young leaves. These findings indicate that PDA medium is more productive for the isolation of endophytic fungi from *O. taihangensis* than the other three culture media. Additionally, the presence of endophytic fungi in old leaves is over three times

higher than that in young leaves. In the test the effectiveness of surface disinfection, absence of any growth on spread plate indicated that surface disinfection was effective and the fungi were endophytic.

A standard curve was generated using mass concentration (mg/ml) of quercetin, rutin, kaempferol, and isorhamnetin as the abscissa (X) and the peak area values as the ordinate (Y). These were respectively as follows: $Y = 186524X - 79.32$, $R^2 = 0.9947$; $Y = 41823X - 45.8$, $R^2 = 0.995$; $Y = 192350X - 161.3$, $R^2 = 0.9999$; and $Y = 109914X - 161.3$, $R^2 = 0.9999$. Five different routine HPLC standards showed different peak periods, where that of quercetin was 16.134 - 18.347 min, rutin was 5.277 - 5.744 min, kaempferol 27.208 - 29.043 min, and isorhamnetin is 33.42 - 36.344 min.

Table 2 shows the results of the 5 colorimetric assays indicated isolates T1, T2, T8, T9, S8, S10, R1, and R2 as flavonoid-producing endophytic fungi. All eight isolates produced various types of flavonoids, except for kaempferol, which was not detected in any sample. The results showed that T1, T8, and R1 only produced rutin and do not contain other kinds of flavonoids, and T2 only generated quercetin. In addition, R2 produced both rutin and quercetin, and T9, S9, and S10 isolates produced three flavonoids: quercetin, rutin, and isorhamnetin, had a maximum yield of 0.0101 mg/ml, 0.0398 mg/mL, and 0.0475 mg/ml, respectively. Although T10, S2, and Q2 showed three kinds of positive color reactions, these were not designated as flavonoid-producing isolates because no distinct color reaction was observed. Subsequent trials did not show that flavonoids were produced (Table 2, not shown).

Mycelial samples of T1, T2, T8, T9, S8, S9, S10, R1 and R2 were subjected to the flavonoid color reaction assay. We extracted the mycelial inclusions by grounding the dry mycelia using a sterile mortar into a powder, and soaking the powder overnight with leach liquor (methanol: concentrated hydrochloric acid = 7:3) at a solid-liquid ratio 1:20. Then, the mixture was subjected to ultrasonication at a setting of 35% power for 1 h. The samples were centrifuged at 12,000 rpm for 10 min and then vacuum-dried in a rotary evaporation apparatus to obtain a 20-folds enriched extract. Finally, the samples were diluted in water to a final volume of 10 ml. The results showed that except for isolate S8, the other isolates generated three or more positive results using five different chromogenic reactions. Therefore, we determined that endophytic fungal samples T1, T2, T8, T9, S9, S10, R1, and R2 produced flavonoids. Moreover, we analyzed the type and amount of flavonoids in the rest of the eight isolates by HPLC. The above results have been given in Table 2.

The colony and microscopic characteristics of the 8 endophytic fungal isolates are shown in Plate 1. T1 (*Fusarium*): Colony is cotton-like, with a light yellow color, of which the back is pink. White villous hyphae, a diaphragm can be observed under a microscope, located close to new colonies, intensity declines from the center to the edge. Conidium is colorless, bent on both ends are typically sickle-shaped. T2 (*Fusarium*): Colony is white; the center of the colony at the side facing the agar is yellowish to brown in color. Hairy hyphae with colorless, and developed. Possess sickle-shaped and elliptical conidia, in chain-like structures. T8 (*Alternaria*): Colonies are dark bluish gray and felty. Conidiophores are dark, without branches. Conidium is dark with diaphragms, mediastinum membranes, and beaks. T9 (*Aspergillus*): Ash black colony in color, mycelium possesses a diaphragm. Apex of conidiophore is spherical in shape and black in color, surrounded by black spores. S9 (*Colletotrichum*): Colony with villous and with a smooth edge, mature butterfly-shaped conidia are visible. Conidia are ovoid, with both ends being obtuse, colorless, and consisting of cellular units. Conidiophore is simple and elongated. S10 (*Pestalotiopsis*): Distinct acervuluslocates beneath a layer of cuticle. Simple and elongated conidiophore, Ovoidconidium, both ends being obtuse, colorless, and consists of cellular units. R1 (*Penicillium*): Colony, cinerous and clings to the culture medium during growth. Conidiophore, slender, the ends of branches have small terriers. Conidiophore and conidium, both cyan in color,

broom-shaped. R2 (*Alternaria*): Colony, cinerous, felty. Conidiophore, unbranched simple and dark, also with simple chains of conidia. Conidium, dark-colored, beaked, with diaphragms and media stinum membranes (Barnett and Hunter 1972).

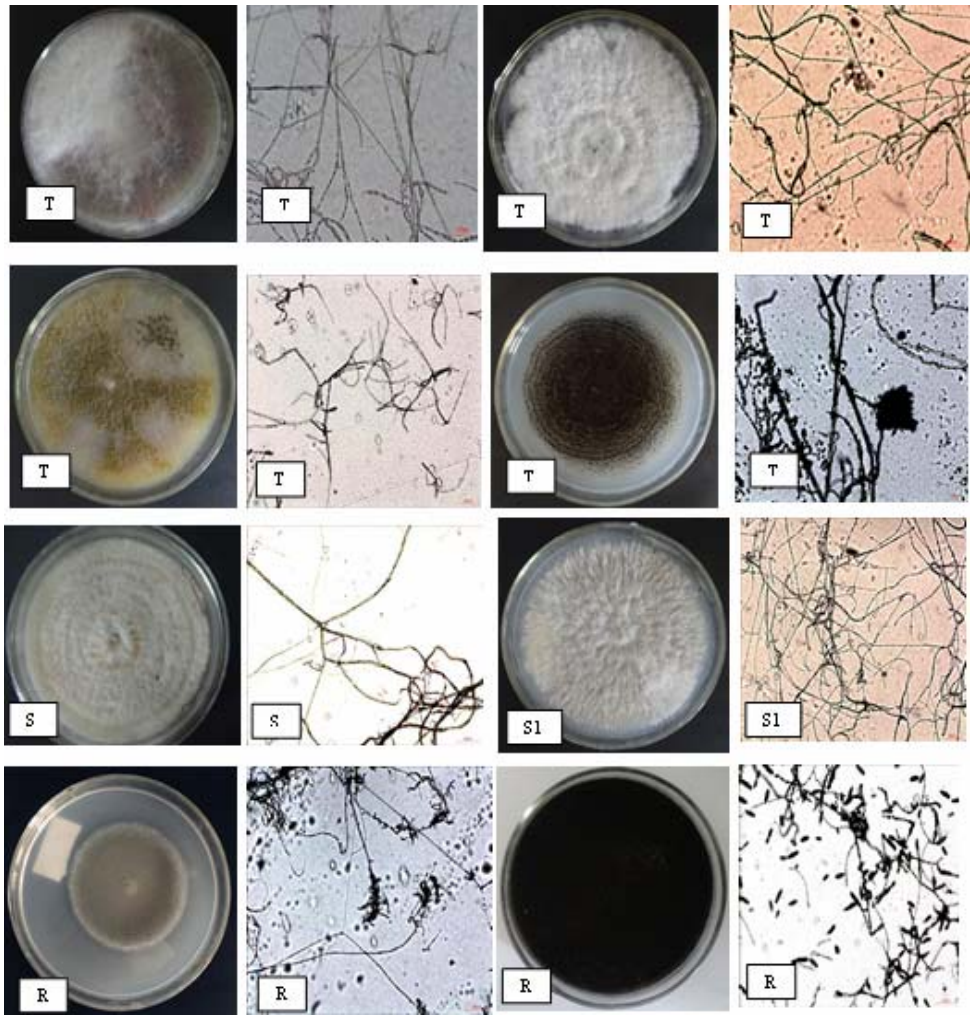

Plate 1. Colony and micrographs of endophytic fungi T, S9, S10, R1 and R2.

In the molecular identification of endophytic fungi, the length of the ITS fragments of the eight endophytic fungi were as follows: T1 (516 bp), T2 (506 bp), T8 (543 bp), T9 (562 bp), S9 (542 bp), S10 (538 bp), R1 (521 bp), and R2 (553 bp) and The ITS sequence of the eight isolates were as identified.

BLAST analysis of the ITS sequences of the endophytic fungi was performed to identify homologous sequences, which are presented as follows (Table 2). The ITS sequences of T1, T2, T8, T9, S9, S10, R1, and R2 were closely related to *Fusarium oxysporum*, *Fusarium*, *Alternaria*, *Aspergillus*, *Colletotrichum*, *Pestalotiopsis*, *Penicillin* and *Alternaria,* respectively, as indicated by

the topology of the reconstructed evolutionary tree (Fig. 1). These findings were consistent with the results of morphological identification. Therefore, the authors confirmed the identity of endophytic fungal isolates as *Fusarium* (T1 and T2), *Alternaria* (T8 and R2), *Aspergillus* (T9), *Pestalotiopsis* (S10), *Penicillin* (R1), *Colletotrichum* (S9).

Table 2. Flavonoid content of various isolates.

Isolates	Quercetin (mg/ml)	Rutin (mg/ml)	Kaempferol (mg/ml)	Isorhamnetin (mg/ml)
T1	-	0.0331 ± 0.0034^{b}	-	-
T2	0.0071 ± 0.0017^{b}	-	-	-
T8	-	0.0127 ± 0.0026^{c}	-	-
T9	0.0059 ± 0.0029^{b}	0.0354 ± 0.0041^{b}	-	0.0366 ± 0.0075^{a}
S9	0.0116 ± 0.0034^{a}	0.0374 ± 0.0055^{b}	-	0.0365 ± 0.0053^{a}
S10	0.0087 ± 0.0015^{ab}	0.0358 ± 0.0043^{b}	-	0.0460 ± 0.0047^{a}
R1	-	0.0821 ± 0.0025^{a}	-	-
R2	0.0068 ± 0.0016^{b}	0.0150 ± 0.0018^{c}	-	-

- indicates not detected. a,b,c = Indicate the significant difference at 0.05 level.

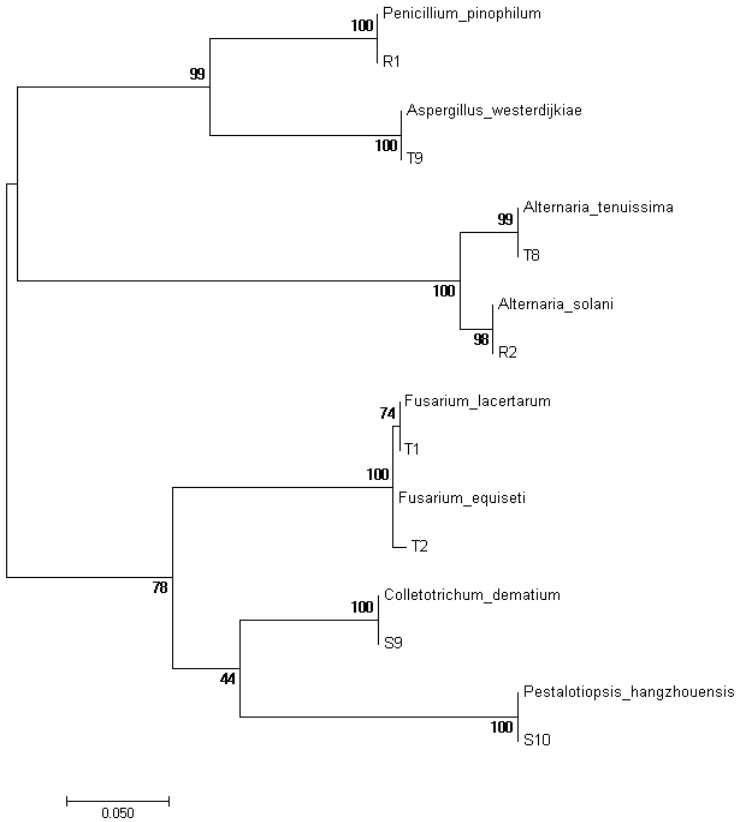

Fig. 2. Phylogenetic trees of the eight *Opisthopappus taihangensis* endophytic fungi isolates based on its 5.8 rDNA-ITS sequence.

These were set based on ML method and level of the genus. And the numbers on the tree refered to support rate of 1000 times repeated sampling on the branch with *Diplodia seriata* as an outgroup.

As far as known this is the only study in which flavonoid-producing endophytic fungi from *O. taihangensis* leaves has been isilated. The flavonoids produced by the endophytic fungi were analyzed by HPLC. It was observed that flavonoid-producing endophytic fungi are present in the leaves of the medicinal plant *O. taihangensis*.

Table 3. Results obtained by BLAST analysis of homologous sequences for flavonoid-producing endophytic fungi isolates from *Opisthopappus taihangensis.*

Isolates	Similar ITS sequence' genbank accession number	Species	Score	% similarity/ident.	E-value
T1	JX391935.1	*Fusarium lacertarum*	915	96	0.0
T2	KY365589.1	*Fusarium equiseti*	915	98	0.0
T8	KX065003.1	*Alternaria tenuissima*	968	99	0.0
T9	KP689263.1	*Aspergillus westerdijkiae*	998	99	0.0
S9	KJ425579.1	*Colletotrichum dematium*	1,002	100	0.0
S10	AY526871.1	*Pestalotiopsis hangzhouensis*	977	99	0.0
R1	JQ003471.1	*Penicillium pinophilum*	957	99	0.0
R2	KT721914.1	*Alternaria solani*	1,022	100	0.0

In 1993, strobel isolated the taxol-producing endophytic fungi *Taxomyces andreanae* from the bulk of fir trees. Thereafter, researchers have focused on isolating medicinal secondary metabolites from various endophytic fungi. By the 21st century, investigations on endophytic fungi in medicinal plants had gained global attention. A number of points should be addressed in future studies. First, it is essential to determine whether the flavonoids produced by endophytic fungi are the same as those produced by the host plant by analyzing their chemical formulas. In addition, methods aiming at increasing the flavonoid yield of endophytic fungi should be developed for large-scale production.

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