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Full Length Research Paper

Isolation of endophytic fungi from *Tripterygium wilfordii* and their insecticidal activities

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In order to solve the insufficient supplement of important medicinal and agricultural plant *Tripeterygium silfordii*, the endophytic fungi which may produce active compounds were studied in this work. 86 endophytic fungi strains from plant *T. wilfordii* were isolated and their larvicidal activities were studied in this paper. Morphological observation preliminarily indicated that these fungi belong to 10 genera, in which *Fusarium*, *Pesalotiopsis*, *Alternaria* and *Thizoctonia* are the dominant flora, accounting for 24.4, 14.0, 11.6 and 10.5% of the total isolated strains, respectively. The metabolites from 86 strains were further extracted to determine their ability to kill mosquito larvae. The results indicate that the ethyl acetate extracts of 16 strains' mycelium exhibit Larvicidal activity, accounting for 18.6% of the isolated strains; and the ethyl acetate extracts of fermentation broth for nine strains (10.5%) showed the larvicidal activity. The active strains were focused in genera *Aspergillus*, *Penicillium*, *Phoma* and *Pesalotiopsis*. In addition, the ethyl acetate extracts of one stain's mycelium contained 8.03 µg wilforgine per gram dry mycelium weight in HPLC analyze, which was further grouped into *Pestalotiopsis malicola* based on the morphological and phylogenetic analysis of its internal transcribed spacer (ITS) sequencing.

Key words: *Tripterygiun wilfordii* Hook, endophytic fungus, insecticidal activity, internal transcribed spacer (ITS) sequence, alkaloids.

INTRODUCTION

Endophytic fungi reside within internal tissues of living plants without visibly harming the host plant (Schulz et al., 2002). Some endophytic fungi have been recognized as a repository of novel compounds of immense value in agriculture, industry and medicine (Naik et al., 2008). To date, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been obatined from the endopytic fungi (Verma et al., 2009; Aly et al., 2010; Yu et al., 2010; Kharwar et al., 2011).

Tripterygium wilfordii Hook f., a perennial twinning vine of the family Celastraceae, is a traditional Chinese herbal medicine and an important insecticidal plant (Kumar et al., 2004). Till now, more than 70 components, including triterpenes, glycosides, diterpenes, sesquiterpenes, quinones and alkaloids with a variety of biological activities, have been isolated from plants in the Genus *Tripterygium* (Kutney et al., 1992; Brinker et al., 2007; Tao and Lipsky, 2000). Indeed, several studies have demonstrated that some compounds extracted from the *T. wilfordii* showed strong antifeedant action, insecticidal action, insect narcosis, anticancer action and immunosuppressive functions, widely used in medicine and agriculture (Luo et al., 2004; Liu, 2011; Xia and Chen, 1990). However, like other active phytochemical compound, the content of medicinal substance in *T. wilfordii* is extremely low and wild resources of *T. wilfordii* are rare, while artificial cultivated *T. wilfordii* grows slowly and needs long harvest period, the production of *T. wilfordii* is unable to meet market demand. In order to solve resource shortage of *T. wilfordii*, we systematically isolated its endophytic fungi and preliminarily studied their taxa and distribution, and analyzed the active substances from those with insectici-

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dal activity, hoping to obtain same insecticidal substances with *T. wilfordii* in order to ease the situation of lacking supply resources of medicinal plant *T. wilfordii* and to meet the market demand.

MATERIALS AND METHODS

Sample collection

In July 2010, the fresh root, stem, and leaf samples of *T. wilfordii* were collected from Taining Mountainous area in Fujian to keep postharvest moisture and ensure the activity of endophytic fungi.

Culture medium

The isolation, purification and preservation of endophytic fungi were performed on potato dextrose agar (PDA) plates supplemented with 50 μg/mL streptomycin and 40 μg/mL chloramphenicol and potato dextrose broth (PDB).

Isolation of endophytic fungi

Sample surface was disinfected according to Zeng et al. (2008). In detail, fresh root, stem, and leaf samples were washed with tab water, cut into small pieces of 3 cm × 3 cm, placed in 75% ethanol for 2 min, rinsed 3 times with sterile water, then soaked in 0.1% mercuric chloride for 8 min and washed with sterile water for four times. Then the samples were further cut into small pieces of 0.2 cm × 0.2 cm, placed under aseptic condition on PDA plate containing 50 μg/mL streptomycin and 40 μg/mL chloramphenicol, cultured upside down at 25°C for 20 days. The growth of endophytic fungi was observed daily. Newly grown mycelia were picked up and transferred onto new PDA plates and further purified till pure strains were obtained. The last washing sterile waters were plated on PDA plates and cultured under the same condition as controls to ensure thorough surface sterilization.

Morphological identification of endophytic fungi

Fungi were primarily identified based on their morphological features described in the "Fungi Identification Manual" (Wei, 1979). Their cultural characters were observed using point inoculation method. In brief, a small amount of mycelium was picked up with a vaccination needle, point planted in the center of PDA plate and then cultured at 25°C for a certain period of time to observe their colony size, color, texture, edge, shape and the presence of exudate and odor. The mycelium and spore characteristics were observed under a microscope, and their diameter and size were measured using a microscopic micrometer.

Flask fermentation of endophytic fungi and extraction of their metabolites

Under sterile conditions, 10 cakes of purified well-growing fungal colonies were obtained by punching their edge with a 4 mm diameter puncher. The cakes were placed into 250 mL conical flasks with 50 mL PDB and cultured in a shaker at 25°C and 120 rpm for 10 days.

Fermentation products were centrifuged at 5000 rpm for 20 min to collect the fungi-free fermentation broths and fungal mycelia. The obtained mycelia were freeze-dried. 2 g dry mycelia was placed in a flat-bottom tube, sonic extracted by 15 mL ethyl acetate for 10 min for three times. The extracts were combined, concentrated under vacuum and used as mycelium extract samples for further investigation.

The sterile fermentation broths were extracted with one-third volume of ethyl acetate for three times. The combined extracts were concentrated under vacuum and used as extracellular metabolic extracts for further analysis.

Measurement of larvicidal activity

Larvicidal activity was measured using the immersion method as reported previously (Wu et al., 2010). The tested mosquitos were the third instar larvae of *Culexpipiens pallens* fed in an indoor environment with relative humidity of 70 to 80%, photoperiod of L:D=16 h:8 h and temperature of 24 to 26°C. The extracellular metabolic extract samples and mycelium extract samples were first fully dissolved in 20 μL dimethyl sulfoxide (DMSO) and then diluted to final concentration of 1 mg/mL. 2% DMSO water solution was used as control. Each treatment was performed using 30 larvae and repeated for three times. The numbers of dead and live larva were counted 24 h after treatment.

Mortality (%) = (The number of dead larva / the number of total tested larva) × 100

Corrected mortality (%) = (the number of dead larva in treatment group - the number of dead larva in control group)/the number of total tested larva × 100

Examination of tripterygium alkaloids

Tripterygium alkaloids were examined using Hypersil BDS C18 column (4.6 mm×250 mm, 5 μm). In detail, 20 μL sample was injected into the column pre-warmed to 30°C, and eluted with 5% to 70% acetonitrile gradient for 20 min, 70% acetonitrile for 10 min, 70 to 5% acetonitrile/water for 5 min, and 5% acetonitrile for 5 min at flow rate of 1.0 mL/min. The eluent was detected by absorption at 220 nm and qualitatively and quantitatively compared to the retention times and peak areas of the mixed standards triptolide, wilforgine and wilforine, respectively. The concentration of tripterygium alkaloids was calculated as:

$C_{sample}=C_{standard} \times A_{sample}/A_{standard}$

$C_{mycelia} = C_{sample} \times V_{sample}/V_{fermentation\ broth} \times M_{mycelia}$

Where $C_{standard}$ is standard concentration (mg/mL), $A_{standard}$ is the area of standard peak, A_{sample} is the area of sample peak and C_{sample} is the alkaloid concentration of each sample.

DNA sequencing and phylogenetic analysis of alkaloids production strain

Fungal DNA was extracted using a Fungal DNAout Kit (OMEGA D3390-01,USA). 5.8S rDNA was PCR amplified using 2 oligonucleotide primers: ITS1 (5'-TC CGTAGGTGAACCTGCGG-3') and ITS4(5'-TCCTCCGCTTATTG ATATG C-3'). Final concentrations for 25 μL PCR amplification reactions were performed by adding 1 μL DNA to 25 μL of reaction mixture: 50ng/μL of template DNA, 2.5μL of 10×PCR reaction buffer, 20pmol primers of ITS1 and ITS4, and Taq PCR Master Mix Kit(Biomiga, USA). The PCR temperature profile used was: 95 s at 94°C followed by 35 cycles of 35 s at 94°C, 60 s at 52°C, 60 s at 72°C and a final extension at 72°C for 10 min. PCR products of the expected size were revealed on 1% agarose gels containing ethidium bromide (10ug/ml). The purification and detection of PCR production were carried by the company

Table 1. The composition of endophytic fungi in *T. wilfordii.*

Phylum	Class	Order	Family	Genus	Number of strain	Proportion %
Deuleromy-cotina	Hyphomycetes	Moniliales	Moniliaceae	Penicillium	3	3.5
				Aspergillus	6	7.0
			Dematiaceae	Alternaria	10	11.6
				Curvularia	8	9.3
	Coelomycetes	Tuberculariales	Tuberculariaceae	Fusarium	21	24.4
		Amphisphaeriales	Amphishaeriaceae	Pesalotiopsis	12	14.0
				Colletotrichum	7	8.1
		Sphaeropsidales	Sphaeropsidaceae	Phoma	2	2.3
		Mycellia sterilia	Agonomycetaceae	Thizoctonia	9	10.5
Ascomycotina	Pyrenomycetes	Sphaeriales	Sordariaceae	Sordaria	3	3.5
Unidentified					5	5.8

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Sequence was submitted to GenBank for BLAST analysis; similar sequences were chosen, and MEGA 4.0 software and Kimura-2 model were used for the construction of neighbor-joining (NJ) phylogenetic tree, [the value of Bootstrap≥50% (1000 replication)].

RESULT AND DISCUSSION

Isolation of endophytic fungi in *T. wilfordii*

A total of 86 endophytic fungal strains were isolated from T. *wilfordii* root, stem and leaf tissues. Among them, 81 were morphologically identified into 10 genera, 7 families, 6 orders, 3 classes, and 2 subphylums primarily. As shown in Table 1, *T. wilfordii* are rich in endophytic fungal species. At class level, 55.8% of the isolated strains are in class Hyphomycetes, 34.9% in class Coelomycetes and only 3.5% in class Pyrenomycetes of subphylum ascomycetes. At order level, Moniliales, Tuberculariales and Amphisphaeriales are dominant flora, accounting for 31.4, 24.4 and 22.1%, of the isolated strains, respectively. At genus level, *Fusarium* is the dominant, accounting for 24.4% of the total isolated strains, followed by *Pesalotiopsis*, *Alternaria* and *Thizoctonia*, accounting for 14.0, 11.6 and 10.5%, respectively. Except Sordaria which belonged to suborder ascomycetes, all other fungi belonged to suborder imperfect.

We also found that different endophytic fungi were isolated from different parts and different tissues of *T. wilfordii* and their isolation frequency was different. As shown in Table 2, the order of isolation frequency of endophytic fungi in *T. wilfordii* from high to low was roots, stems, and leaves. A total of 44 endophytic fungi were isolated from roots, accounting for 51.2% of the total isolated strains. These fungi belong to nine genera. A total of 26 endophytic fungi were isolated from stems, accounting for 30.2%. These fungi belong to nine genera. A total of 11 endophytic fungi were isolated from the leaves, accounting for 12.8%. These fungi belong to six genera. Distribution of endophytic fungi in different genera and species indicated that strains in genus Fusarium were distributed in roots, stems, and leaves, accounting for 27.3, 23.1 and 27.3 of the total strains isolated from roots, stems and leaves, respectively, indicating that the genus Fusarium is the dominant in *T. wilfordii*.

Larvicidal activity of endophytic fungi in *T. wilfordii*

The third instar larvae were used to measure the larvicidal activity of the 86 endophytic fungi isolated from *T. wilfordii*. As shown in Table 3, the mycelium ethyl acetate extracts of 16 endophytic fungi and the fermentation broth extracts of 9 endophytic fungi had larvicidal activity, accounting for 18.6 and 10.5% of the total isolated strains, respectively. Among them, the mycelium extract or the fermentation broth extract of three endophytic fungi had 100% larvicidal activity. Overall, the major active strains were *Aspergillus*, *Penicillium*, *Phoma* and *Pesalotiopsis*.

Content of tripterygium alkaloids in the metabolites of active strains

We measured the content of tripterygium alkaloids in the metabolites of the 22 strains with larvicidal activity. As shown in Figure 1, 1 g ethyl acetate extract was dissolved in 0.5 mL acetonitrile and subjected to HPLC analysis. Compared to 0.2 mg/mL standard triptolide, wilforgine and wilforine, which had retention time and peak area of 15.518 min and 19,986,959 (mAU*s), 21.573 min and 678 188 (Mau*s), as well as 22.798 min and 10459546 (Mau*s), respectively, the sample had a peak with retention time of 21.632 min, which is similar to the standard wilforgine, and area of 544 703 (mAU*s), suggesting that active endophytic fungi in *T. wilfordii* contain are able to generate wilforgine. Its content was 16.06 μg/mL, that is 8.03 μg per gram of dry mycelium weight.

Table 2. The distribution in number and species of endophytic fungi in *T. wilfordii.*

Genus	Root	Stem	Leaf
Penicillium	2	1	0
Aspergillus	4	2	0
Alternaria	5	3	2
Curvularia	3	3	2
Fusarium	12	6	3
Pesalotiopsis	6	4	2
Colletotrichum	4	3	0
Phoma	0	1	1
Thizoctonia	5	3	1
Sordaria	3	0	0

Table 3. Toxic activity of endophytic fungi isolated on *T. wilfordii* against the 3th instar larvae of *C. pallens.*

Corrected mortality (%)	Mycelial extraction (1 mg/mL)		Fermentation extraction(1 mg/mL)	
	Strain number	Percentage in total tested strains (%)	Strain number	Percentage in total tested strains (%)
++++	3	3.5	2	2.3
+++	3	3.5	1	1.2
++	1	1.2	2	2.3
+	9	10.5	4	4.7

Time of treatment was 24 h; ++++, corrected mortality≥90%; +++, 70-90%; ++, 50-70%; +, 0-50%.

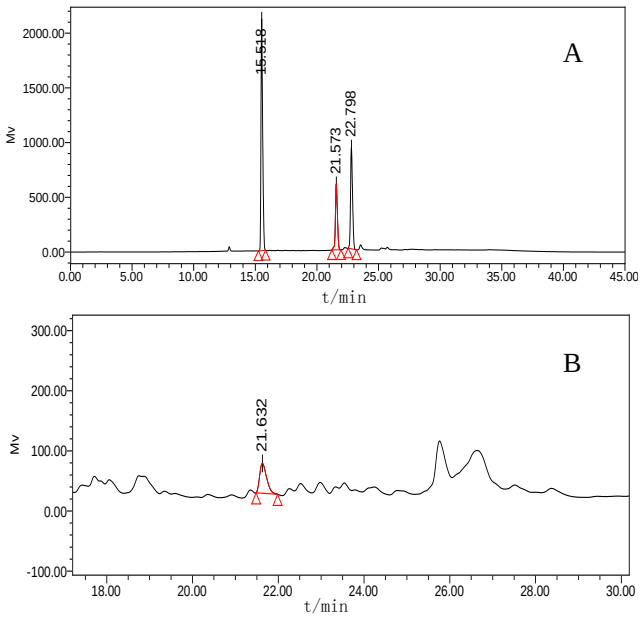

Figure 1. Spectra of High performance liquid chromatography. **A.** Standard samples of tripterygium alkaloids. **B.** mycelium ethyl acetate extract of Endogenous fungus.

Figure 2. Growth of 4-3 on PDA (left panel) and its conidia (right panel).

Sequence and genetic tree analysis of active strain 4-3

Morphological analysis indicated that strain 4-3 may belong to the genera of *Pestalotiopsis* (Figure 2). In microscopy observation, the aerial mycelium is thin and grey on PDA medium. Conidia is coryneform; have three apical setulae and five cell. Both the basal and apical cells are hyaline, and the median three cells are dark brown. The 5.8S rDNA sequence of 4-3 was compared to those of other *Pestalotiopsis* species (GeneBank database). The sequence has a 99% identity to that of species from the genus *Pestalotiopsis*, also suggesting that strain 4-3 belong to the genus *Pestalotiopsis* (>97%).

Phylogenetic analysis based on ITS-5.8S rDNA sequence revealed that strain 4-3 and *P. malicola* (JF501649) were clustered in close clades (Figure 3), and had 99% identity.

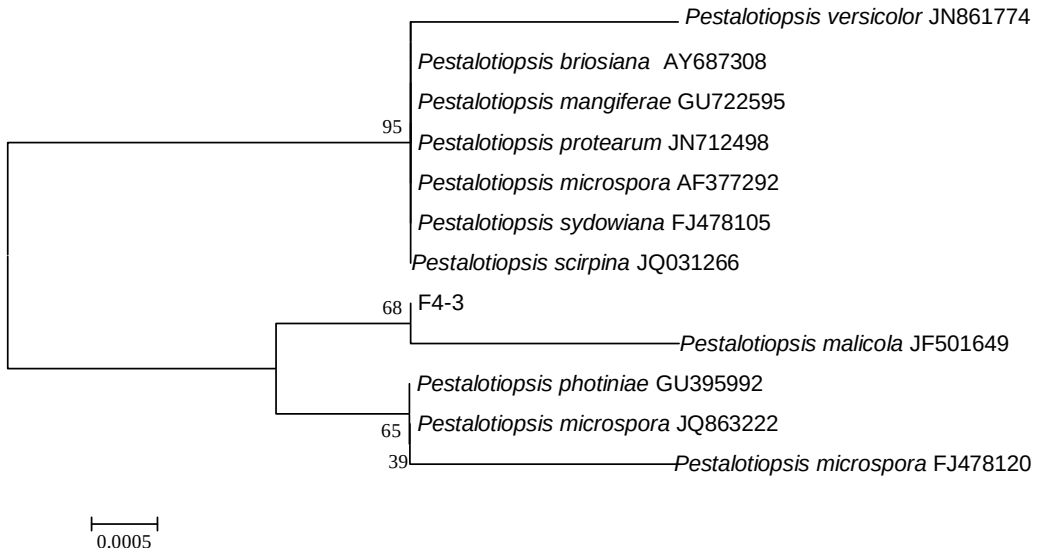

Figure 3. Phylogenetic tree of strain 4-3.

DISCUSSION

Since the discovery of the first anticancer compound-Taxol-could be biosynthesized by endophytic fungi, interest in endophytes as potential producers of novel, and biologically active products has increased (Hazalin et al., 2009; Zhao et al., 2012). To date, the bioactive compounds production of endophytic fungi from *Taxusyunnanensis*, *Catharanthus roseus*, *Camptotheca acuminata*, *Podophyllum hexandrum*, *Canada thistle* and other precious plants have been reported (Nadeem et al., 2007; Cragg and Newman, 2009; Yang et al., 2010; Zhang et al., 2000). In our study, a total of 86 endophytic fungi were isolated and were assigned to taxa. Among them, fermentation broth or mycelium for 25 (29%) isolates showed the larvicidal activity. In addition, the strain 4-3 with the highest wilforgine production of 8.03 µg/g, was identified as *P. malicola.* Beroza reported that the major insecticidal components of *T. wilfordii* are alkaloids, among which, wilfordine wilforine, wilforgine, and celacinnine have strong insecticidal activity (Beroza, 1953). In conclusion, this preliminary screening of endophytic fungi revealed their potential to yield potent bioactive compounds for drug discovery and making microbial fermentation possible to produce more tripterygium alkaloids. It also provides solutions to solve the bottleneck issues such as low content of tripterygium active components, slow growth, and lack of natural resources.

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