Antibacterial activity of the endophytic fungi from a traditional Chinese herb *Paris polyphylla* var. *chinensis*

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Accepted 27 February, 2012

A total of 29 endophytic fungal isolates were separated from the healthy rhizomes of *Paris polyphylla* var. *chinensis* (Trilliaceae), which is a traditional medicinal herb mainly distributed in central China. Eight distinct isolates (that is, Papochf01-Papochf08) were selected for further taxonomical identification by morphological traits and internal transcribed spacer (ITS) rRNA gene sequence analysis. The results based on morphological characters revealed six genera namely *Bionectria* (Papochf04), *Fusarium* (Papochf05), *Leptodontidium* (Papochf07), *Neonectria* (Papochf02), *Setophoma* (Papochf08) and *Trichocladium* (Papochf01). Isolates Papochf01, Papochf02, Papochf03, Papochf06 and Papochf08 have not been previously reported as the endophytic fungi. The filtrate and mycelia n-butanol extracts obtained from the isolates Papochf05 and Papochf08 exhibited strong inhibition on all the test bacteria including *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas lachrymans*, *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Salmonella typhimurium*, and *Xanthomonas vesicatoria*. This finding shows that endophytic fungi from *P. polyphylla* var. *chinensis* could be an alternative source for producing antimicrobial agents.

**Key words:** *Paris polyphylla* var. *chinensis*, endophytic fungi, n-butanol extract, TLC-bioautography assay, antibacterial activity.

**INTRODUCTION**

Plant endophytic fungi are defined as the fungi which spend the whole or part of their lifecycle colonizing inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease (Rodriguez et al., 2009). They have been found to produce many valuable bioactive metabolites including anti-microbial, anti-insect, anti-cancer, anti-diabetic and immuno-suppressant compounds with their great potential applications in agriculture, medicine and food industry (Verma et al., 2009; Zhou et al., 2010; Kharwar et al., 2011; Zhao et al., 2011a). The typical bioactive compounds included taxol from endophytic fungus *Taxomyces andreanae* in *Taxus brevifolia* (Stierle et al., 1993), podophyllotoxin from *Phialocephala fortinii* in *Podophyllum peltatum* (Eyberger et al., 2006), camptothecin from *Entrophospora infrequens* in *Notaphytes foetida* (Puri et al., 2005), spirobisnaphthalenes from endophytic fungus Dzfl2 in *Dioscorea zingiberensis* (Cai et al., 2009; Zhao et al., 2011b), beauvericin from *Fusarium redolens* Dzfl2 in *D. zingiberensis* (Xu et al., 2010), helvolic acid from *Pichia guillermondi* in *Paris polyphylla* var. *yunnanensis* (Zhao et al., 2010), and botralin from *Hyalodendriella sp.* Ponipodef12 in poplar hybrid ‘Neva’ (Zhong et al., 2011a). *Paris polyphylla* Smith var. *chinensis* (Franch.) Hara (Trilliaceae), a perennial endangered medicinal herb, is mainly distributed in central China (Yuan et al., 2004). It has been used as a traditional Chinese medicine (TCM) for a long time with a variety of medicinal uses as the antidote for snake bite, antibiotic, antitumor, contraceptive, sedative, and so on (Mimaki et al., 2000). The phytochemical and pharmacological studies on this herb have resulted in isolation of several steroidal

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saponins with a wide range of biological activities including cytotoxic activity on HL-60 cells (Mimaki et al., 2000), haemostatic effect (Ma and Lau, 1985) as well as antifungal activity on Cladosporium cladosporioides and Candida sp. (Deng et al., 2008). To the best of our knowledge, there is no reported study on the endophytic fungi associated with P. polyphylla var. chinensis though there were some reports about the endophytic bacteria isolated from this plant (Zhao et al., 2005; Zhang et al., 2006). The aim of this study was to isolate and identify the endophytic fungi from the rhizomes of P. polyphylla var. chinensis as well as to screen their antibacterial activity.

MATERIALS AND METHODS

Plant materials

The healthy rhizomes of three-year-old Paris polyphylla Smith var. chinensis (Trilliaceae) were collected from the Shennongjia Forest District in Hubei Province of China in June 2009. The plant was identified according to the morphological features by Prof. Jiaru Li, a botanist from the College of Life Sciences at Wuhan University of China. The voucher specimen (BSMPSI-200906001) of this plant was deposited in the Herbarium of the Institute of Chinese Medicinal Materials, China Agricultural University. The plant samples were stored in the sealed plastic bags at 4°C until required.

Isolation and culture of the endophytic fungi

The isolation of fungi was performed following the process described by Xu et al. (2008) with some modifications. Briefly, the healthy rhizomes of P. polyphylla var. chinensis were washed thoroughly with running tap water first, then surface sterilized by dipping them in 75% ethanol for 30 s, followed by immersing in 0.2% mercuric chloride for 20 min, then rinsed in sterile distilled water thrice (that is, 5 min for each time), and finally dried on the sterile tissue paper. The epidermis of each rhizome explant was confirmed by placing the sterile epidermal tissues on potato dextrose agar (PDA) plate. All flasks were incubated on a rotary shaker at 150 rpm and 25°C for 15 days. For identification, all the bacterial strains were individually placed on PDA plates supplemented with streptomycin sulfate (500 mg/L) to suppress bacteria growth. After the plates were incubated in the dark at 25°C for 7 to 30 days, the number of fungi was counted, and each fungal colony was isolated from the colony with clear edges. The concentrated methanol extract was dissolved in 0.2% mercuric chloride for 20 min, then rinsed in sterile distilled water thrice (that is, 5 min for each time), and finally dried on the sterile tissue paper. The epidermis of each rhizome explant was confirmed by placing the sterile epidermal tissues on potato dextrose agar (PDA) plate. After sterilization, each rhizome (without epidermis) was cut approximately into 5 × 5 × 5 mm cubes which were individually placed on PDA plates supplemented with streptomycin sulfate (500 mg/L) to suppress bacteria growth. After the plates were incubated in the dark at 25°C for 7 to 30 days, the number of fungi was counted, and each fungal colony was isolated and sub-cultured to get a pure culture. The colonization frequency (CF) of each endophyte was calculated according to the method of Hata and Futai (1995). 

\[
CF = \left( \frac{N_{col}}{N} \right) \times 100, \quad \text{where } N_{col} \text{ is the number of cubes colonized by each fungus and } N \text{ is the total number of cubes.} 
\]

All the isolated fungi were deposited at the Department of Plant Pathology, China Agricultural University.

Morphological characterization

The morphological characters including colony diameter, texture, color, the dimensions and morphology of hyphae and conidia of the fungal isolates were observed and described according to the methods of Phoita et al. (2005), Barnett and Hunter (1972) and Ainsworth et al. (1973).

DNA extraction, ITS-DNA amplification and sequence analysis

Total genomic DNA of the fungal isolates was prepared according to a modification of the rapid preparation of DNA from filamentous fungi (Raeder and Broda, 1985). Primers ITS1 (5'-TCCGTAAGTTGAACCTGCGG -3') and ITS4 (5'-TCTCCGCTATTGATATGC -3'), as well as ITS-DNA amplification were referenced by our previous reports (Xu et al., 2008; Li et al., 2008; Zhong et al., 2011). For identification, the PCR products were purified using the QIA quick Gel Extraction Kits (Qiagen, Hilden, Germany) and sequenced using the primer pair ITS1 and ITS4 on the ABI PRISM 3730 sequencer. Then the sequences were run by BLASTN program against the database (National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov), and they were submitted to GenBank where the accession numbers were obtained.

Mycelial suspension culture and n-butanol extract preparation

A 1000 ml Erlenmeyer flask containing 200 ml of potato dextrose broth (PDB) was inoculated with 2 to 3 agar plugs containing mycelia taken from the culture of each endophytic fungal isolate purified on PDA. All flasks were incubated on a rotary shaker at 150 rpm and 25°C for 12 h. After suspension culture, the culture broth (1 L for each fungal isolate) was filtrated in vacuum to afford the filtrate and mycelia. The filtrate was extracted with an equal volume of n-butanol for three times. The mycelia were lyophilized and powdered, followed by extracting with ultrasound in methanol for three times. The concentrated methanol extract was dissolved in water, and then extracted with an equal volume of n-butanol for three times. The above n-butanol solutions were concentrated in vacuum at 50°C to obtain mycelia and filtrate extracts, respectively.

Detection of antibacterial activity of the extracts

Thin layer chromatography (TLC)-bioautography assay of the samples was carried out according to the method of Zhao et al. (2008). Three Gram-positive (Bacillus subtilis ATCC 11562, Staphylococcus aureus ATCC 6538 and Staphylococcus haemolyticus ATCC 29970) and five Gram-negative (Agrobacterium tumefaciens ATCC 11158, Escherichia coli ATCC 29522, Pseudomonas lachrymans ATCC 11921, Salmonella typhimurium ATCC14028, and Xanthomonas vesicatoria ATCC 11633) bacteria were selected for antibacterial assay. All these bacterial strains were provided by the Department of Plant Pathology of China Agricultural University. After the TLC plate covered with the test bacteria was incubated at 28°C for 12 h, the color reagent was sprayed with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, purchased from Amresco, USA), and incubated for another 10 min. The presence of antibacterial activity was determined by the formation of well defined inhibition zones made visible by spraying with MTT that was converted to a purple background, and the length of each antibacterial area was measured. All tests were performed in triplicate.

RESULTS AND DISCUSSION

Identification of the endophytic fungi

A total of 29 endophytic fungal isolates were obtained from the healthy rhizomes of P. polyphylla var. chinensis.
According to their morphological features, eight distinct fungal isolates, which colonies growing in Petri dish were shown in Figure 1, were selected for further taxonomical identification with their morphological features shown in Table 1. Six genera namely Bionectria (Papochf04), Fusarium (Papochf05), Leptodontium (Papochf07), Neoneectria (Papochf02), Setophoma (Papochf08) and Trichocladium (Papochf01) were identified. Isolates Papochf03 and Papochf06 were not identified. The fungi of genus Fusarium were the first main isolates with their colonization frequency (CF) as 43.8%. The fungi of genus Setophoma were the second main isolates with CF as 20.8%.

The ITS1-5.8S-ITS4 partial sequences of 8 isolates were submitted to the GenBank to obtain their accession numbers (that is, HQ731629-HQ731636), and the closest related species were got by BLAST analysis (Table 2). The results showed that all the sequences had more than 89% similarity with the species in GenBank. Comparison of the ITS-rDNA sequences obtained from the isolates with the sequences available in the GenBank databases allowed us to analyze the phylogenetic affiliation of these fungi (Figure 2). The molecular characters of the endophytic fungi were basically coincident with their morphological ones. The similarity of Papochf03 was only 94% to Fusarium cuneirostrum AB513850, and considerable morphological differences were found between Papochf03 and Papochf05, which should not be in the genus Fusarium. The isolates Papochf03 and Papochf06 need further identification.

Detection of antimicrobial activity

Antibacterial activity results of the endophytic fungal extracts against eight bacteria by using TLC-bioautography method were shown in Table 3. The mycelia and filtrate n-butanol extracts of some isolates (i.e., Papochf03, Papochf05 and Papochf08) for their inhibitory zones on test bacterium Pseudomonas lachrymans in a TLC plate were shown in Figure 3. Most of the extracts
Table 1. Morphological characters of the endophytic fungi.

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>CF (%)</th>
<th>Partial macro- and microscopic characters</th>
<th>Identified results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papochf01</td>
<td>2.1</td>
<td>Colony cottony, white to gray in the center of colony with aging. Hyphae aseptate, branched, colorless to gray with aging. Dark conidia ovoid or cylindrical, without or with a short conidiophore.</td>
<td><em>Trichocladium</em> sp.</td>
</tr>
<tr>
<td>Papochf02</td>
<td>5.2</td>
<td>Colony cottony, white to yellow in the center of colony with aging. Hyphae septate and branched. Conidia forming in slimy clusters on the colony surface and in the aerial mycelium. Conidia cylindrical, 2– or 3-septate.</td>
<td><em>Neonectria</em> sp.</td>
</tr>
<tr>
<td>Papochf03</td>
<td>8.3</td>
<td>Flat-growing colony, cottony, white hyphae septate. Non-sporulating.</td>
<td>Unidentified</td>
</tr>
<tr>
<td>Papochf04</td>
<td>6.3</td>
<td>Colony white, releasing orange pigment into PDA. Hyphae septate, conidia oval and in a cluster.</td>
<td><em>Bionectria</em> sp.</td>
</tr>
<tr>
<td>Papochf05</td>
<td>43.8</td>
<td>Flat-growing colony, white to pink with aging, releasing reddish yellow pigment into PDA, up to 7.0 cm diam. in one week. Conidia falcate or elliptical.</td>
<td><em>Fusarium</em> sp.</td>
</tr>
<tr>
<td>Papochf06</td>
<td>2.1</td>
<td>Colony cottony, grey to brown in the center of colony, hyphae aseptate. Non-sporulating.</td>
<td>Unidentified</td>
</tr>
<tr>
<td>Papochf07</td>
<td>5.2</td>
<td>Colony gray, moderately grew on PDA. Hyphae dark and septate, and about 5.0–7.5 μm wide hyphae. Non-sporulating.</td>
<td><em>Leptodontidium</em> sp.</td>
</tr>
<tr>
<td>Papochf08</td>
<td>20.8</td>
<td>Colony cottony, releasing red pigment into PDA. Mycelia were hyaline, septate and anastomosing. Pycnidia dark brown to black, subglobose, ostiolate, and occur singly. Conidia continuous, oblong to ovoid and sessile in pycnidia.</td>
<td><em>Setophoma</em> sp.</td>
</tr>
</tbody>
</table>

Table 2. Endophytic fungi and their closest relatives based on the data from BLAST analysis.

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>GenBank accession number</th>
<th>Closest related species</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papochf01</td>
<td>HQ731629</td>
<td><em>Trichocladium</em> opacum FN386299</td>
<td>99</td>
</tr>
<tr>
<td>Papochf02</td>
<td>HQ731630</td>
<td><em>Neonectria</em> macrodidyma AM419075</td>
<td>99</td>
</tr>
<tr>
<td>Papochf03</td>
<td>HQ731631</td>
<td><em>Fusarium</em> cuneirostrum AB513850</td>
<td>94</td>
</tr>
<tr>
<td>Papochf04</td>
<td>HQ731632</td>
<td><em>Bionectria</em> ochroleuca GQ302681</td>
<td>99</td>
</tr>
<tr>
<td>Papochf05</td>
<td>HQ731633</td>
<td><em>Fusarium</em> redolens EF495234</td>
<td>100</td>
</tr>
<tr>
<td>Papochf06</td>
<td>HQ731634</td>
<td><em>Paraphaeosphaeria</em> sp. DQ092522</td>
<td>89</td>
</tr>
<tr>
<td>Papochf07</td>
<td>HQ731635</td>
<td><em>Leptodontidium</em> orchidicola GQ302678</td>
<td>99</td>
</tr>
<tr>
<td>Papochf08</td>
<td>HQ731636</td>
<td><em>Setophoma</em> sacchari FN394730</td>
<td>99</td>
</tr>
</tbody>
</table>

except Papochf07 filtrate extract showed antibacterial activity to some extent. Both *Staphylococcus haemolyticus* and *Salmonella typhimurium* were more sensitive to the extracts than other bacteria. For some fungal isolates (for example, Papochf01-Papochf03, Papochf07), the mycelia extracts showed stronger antibacterial activity than the filtrate extracts. Among the isolates, Papochf05 and Papochf08 exhibited the strongest inhibition on test bacteria. Very interestingly, *Fusarium redolens* has been found to produce beauvericin with a high yield (Xu et al., 2009; 2010). Papochf05, which was identified as *F. redolens* and displayed strong antibacterial activity, should be further studied for its active metabolites.

**Conclusion**

We first reported the endophytic fungi from medicinal plant *P. polyphylla* var. *chinensis*, and detected their antibacterial activity with TLC-bioautography assay. Some fungal isolates (for example, Papochf05 and
Figure 2. Phylogenetic relationship analysis of the isolates Papochf01 to Papochf08. The unrooted tree was generated using ClustalW program by Neighbor-Joining method. Phylogeny test was computed by MEGA 3.1. Bootstrap values (above 50%) from 1000 replicates were indicated at each node.

Table 3. Antibacterial activity of the crude extracts from the endophytic fungi against different bacteria by TLC-bioautography test.

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</tr>
</thead>
<tbody>
<tr>
<td>Papochf01</td>
<td>F</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Papochf02</td>
<td>F</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Papochf03</td>
<td>F</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Papochf04</td>
<td>F</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Papochf05</td>
<td>F</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Papochf06</td>
<td>F</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Papochf07</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
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Table 3. Contd.

<table>
<thead>
<tr>
<th></th>
<th>Papochf07</th>
<th>Papochf08</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Papochf07</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Papochf08</td>
<td>+++</td>
<td>++</td>
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</tbody>
</table>

F, filtrate n-butanol extract; M, mycelia n-butanol extract; A.t., Agrobacterium tumefaciens; B.s., Bacillus subtilis; E.c., Escherichia coli; P.I., Pseudomonas lachrymans; S.a., Staphylococcus aureus; S.h., Staphylococcus haemolyticus; S.t., Salmonella typhimurium; X.v., Xanthomonas vesicatoria; Developing solvent system in TLC was chloroform-methanol (10:1, v/v); -, antimicrobial activity was not observed; +, the length of the antimicrobial activity area was 0 to 1.0 cm; ++, the length of the antimicrobial activity area was 1.0 to 2.0 mm; ++++, the length of the antimicrobial area was more than 2.0 mm; The positive control was streptomycin sulfate which was only sampled on the TLC plate and showed antibacterial activity.

Figure 3. Antibacterial activity screening of the endophytic fungal extracts of Papochf03, Papochf05 and Papochf08 by TLC-bioautography assay. Developing solvent system in TLC was chloroform-methanol (10:1, v/v). The test bacterium was Pseudomonas lachrymans. Antibacterial activity was detected as white inhibition zones against a purple background. A and D were n-butanol extracts of the isolate Papochf08 mycelia and filtrate, respectively. B and E were n-butanol extract of the isolate Papochf05 mycelia and filtrate, respectively. C and F were n-butanol extract of the isolate Papochf03 mycelia and filtrate, respectively.
Papochf08) displayed strong antibacterial activity. The endophytic fungi from *P. polyphylla* var. *chinensis* could be an alternative source for producing antimicrobial agents. Whether the endophytic fungi in *P. polyphylla* var. *chinensis* contribute to the host plant to have antimicrobial activity should be investigated in detail. Further studies on taxonomy and isolation of antimicrobial compounds from these fungi (e.g., Papochf03, Papochf05, Papochf06 and Papochf08) are now in progress.

**ACKNOWLEDGMENTS**

This work was co-financed by the grants from the National Natural Science Foundation of China (31071710 and 30871662) and the Hi-Tech R&D Program of China (2011AA10A202).

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