RESEARCH ARTICLE

Identification of Endophytic Fungi of Medicinal Herbs of Lauraceae and Rutaceae with Antimicrobial Property

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ABSTRACT: This study was conducted to determine taxonomical features and antimicrobial activities of 156 isolates of endophytic fungi collected from twigs of medicinal plants of Lauraceae (67 isolates) and Rutaceae (89 isolates) in central and northern Taiwan. The 156 isolates of fungi were classified into 35 genera in 19 families based on morphological characteristics of mycelia and asexual/sexual spores, as well as molecular phylogenetic analysis of rDNA LSU D1/D2 and ITS regions. The most common endophytes were in the taxa of *Colletotrichum*, *Guignardia*, *Hypoxylon*, *Nigrospora*, *Phomopsis* and *Xylaria*, and the most common hosts were *Citrus* and *Zanthoxylum* of Rutaceae and *Cinnamomum* of Lauraceae. Molecular phylogenetic analysis showed that xylariaceous isolates could be separated into Xylaria and Hypoxylon groups based on rDNA of LSU D1/D2 and ITS regions. Four isolates of endophytic fungi including *Lasmenia* sp. isolate CB10, *Ophioceras* tenuisporum isolate CI02, *Xylaria cubensis* isolate LA04 and *Cyanodermella* sp. isolate TR09 were tested for antimicrobial activities using a dual culture method and *Lasmenia* sp. isolate CB10 and *Cyanodermella* sp. isolate TR09 showed better antimicrobial activity against 12 plant pathogens including 9 fungi and 3 bacteria. Spraying Chinese cabbage (*Brassica rapa*) plants with culture filtrates of the endophytic fungus *Lasmenia* sp. isolate CB10 significantly reduced severity of anthracnose of Chinese cabbage caused by *Colletotrichum higginsianum* under greenhouse conditions. This study suggests that the *Lasmenia* sp. isolate CB10 may be of potential for management of anthracnose of Chinese cabbage.

KEY WORDS: Antimicrobial activity, endophytic fungi, herb plant, Lauraceae, Rutaceae.

INTRODUCTION

Endophytic fungi are capable of living in host plants without causing any symptoms (Petrini et al., 1992). To date, endophytic fungi have been separated into four classes based on host range, type of tissue(s) colonized, colonization in planta, diversity in planta, transmission and fitness benefits (Rodriguez et al., 2009). Moreover, some endophytic fungi may produce secondary metabolites with potential for antimicrobial or anticancer property (Shu et al., 2005; Xu et al., 2008). For example, the endophytic fungus *Taxomyces andreanae* produced taxol with anticancer activity (Stierle et al., 1993). Other reports showed that certain endophytic fungi produced more than twelve metabolites similar to those produced by host plants with therapeutic function, including alkaloids, steroids, terpenoids, isocoumarin derivatives, flavinoids, quinines, phenylpropanoids, phenylpropanoids and ligans, peptides, phenol and phenolic acid, aliphatic compounds and chlorinated metabolites (Li and Liu, 2004; Shu et al., 2005; Strobel, 2003; Wang et al., 2007).

Many plants and algae have been reported to host of fungal endophytes (Davis et al., 2003). Among the host plants, the medicinal herbs are one of the important groups of hosts for endophytic fungi (Huang et al., 2008; Li et al., 2007; Li et al., 2004; Xu et al., 2008; Yan et al., 2007). Previous reports have demonstrated that fungal endophytes from medicinal herbs show efficacy as pharmaceutical and agricultural compounds, especially from Chinese herbs (Kusari et al., 2008; Li et al., 2004; Li et al., 2007; Shentu et al., 2007; Yan et al., 2007; Yi, 2003). Recently, certain isolates of endophytic fungi from Chinese herbs have been used as biocontrol agents for agricultural crops (Backman and Sikora, 2008; Gabler et al., 2010; Kunkel and Grewal, 2003; Maciá-Vicente et al., 2009; Mercier and Jiménez, 2009; Mejía et al., 2008; Redman et al., 1999; Schulz et al., 2002). In this study, endophytic fungi were isolated from two families of medicinal plants, Lauraceae and Rutaceae, collected from natural habitat and arboretum in northern and central Taiwan. Some species of Lauraceae were used for fruits, dyes, medicine and perfume, whereas some species of Rutaceae were used for fruits, medicine, wood and extracted oil. The objectives of this study were to identify endophytic fungi in Lauraceae and Rutaceae in Taiwan and to
Investigate their biodiversity and antimicrobial activities against fungal and bacterial pathogens of plants.

MATERIALS AND METHODS

Plant material and endophytic fungi
Healthy twigs of medicinal herbs of Lauraceae and Rutaceae were collected from Taiwan Seed Improvement and Propagation Station (TSIPS), Taichung, central Taiwan and Fushan Botanical Garden (FBG), Yilan, northern Taiwan (Table 1), 10 twigs/species. Twigs were washed with reverse osmosis (RO) water, cut into segments (3-5 mm), surface-sterilized followed a procedure described by Bills (1996), and inside tissues were took and placed on cornmeal dextrose agar (CDA, Difico, USA) medium supplemented with streptomycin (50 mg/ml) to suppress the growth of bacteria. After incubation at 25°C for 3–5 days, fungi growing out from the segments were transferred onto potato dextrose agar (PDA, Difico, USA) in Petri dishes and each fungus was purified by single hyphal tip isolation. Pure cultures were maintained on PDA slants at 4–10°C for further experiments.

Plant pathogens
Twelve isolates of plant pathogens (9 fungi and 3 bacteria) isolated from diseased plants were used for testing antimicrobial activities of endophytic fungi. They were Alternaria solani AS01 from tomato, Botrytis cinerea NBC12 from strawberry, Colletotrichum gloeosporioides TC01 from yam, C. higginsianum PA01 from Chinese cabbage, Cylindocladiella lageniformis CL01 from loquat, Fusarium oxysporum f. sp. liliifolii Fol-04 from lily, Monilinia fructicola TW01 from peach, Penicillium digitatum DOB02 from orange, Pestalotia psidii TG01 from guava, Pythium aphanidermatum P374 from pea, Erwinia carotovora subsp. carotovora Ec4 and Xanthomonas campestris pv. campestris Xcc17 from cabbage and Ralstonia solanacearum PW2 from tomato.

Morphological and molecular identification of endophytic fungi

A total of 156 isolates of endophytic fungi were selected for morphological and molecular identifications. They were grown on PDA at 25°C under 12-h photoperiod for 10-21 days and examined for colony morphology by naked eye and/or characteristics of asexual spores and/or sexual spores under a compound microscope. In molecular identification, fungal genomic DNA was extracted and prepared from each endophytic fungus by the method of Goodwin and Lee (1993). Species identification of endophytic fungi was performed using the primer pairs NL1/NL4 (5’-GCATATCAATAAGCGGAGGAAAAAG-3’/5’-CTTGTTGCTATTAGAAGGA-3’) for rDNA LSU D1/D2 regions (O’Donnell, 1993) and ITS1/ITS4 (5’-CTTGGTCATTTAGAAGGAAGTAA-3’/5’-TCCTCCGCTTATTGATATGC-3’) for rDNA ITS regions (White et al., 1990). The sequences of individual isolate were blasted with sequence from the GenBank of NCBI/DDBJ/EBML. Amplifications of the D1/D2 and ITS rDNA regions were conducted using 25 μl of PCR reaction mixtures, each containing 1.5 μl total fungal genomic DNA, 0.2 μm of each primer, one unit of Taq DNA polymerase (GeneMark, Taichung, Taiwan) and dNTP mixture (containing 250 μm of each dNTP) and Taq reaction buffer (containing 2 mm MgCl2). For LSU D1/D2 regions amplification, the PCR reaction program was as follows: 95°C for 5 min; 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min; 72°C for 10 min. For ITS regions, the PCR reaction program was as follows: 95°C for 5 min; 35 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 1 min; 72°C for 10 min. The PCR products were run in 1.5% agarose gels containing 0.5 g/ml ethidium bromide in TAE (Tris-acetate, EDTA) buffers. PCR products were purified by spin columns (PCR Clean-Up Kit, GeneMark, Taiwan) and DNA sequencing was performed on an ABI PRISM 3100 automated sequencers (Applied Biosystems, Ramsey Inc., USA).

Phylogenetic analysis of endophytic Xylariaceae

Since endophytic fungi belonging to Xylariaceae were most frequently isolated from samples of medicinal plants collected in this study, isolates of Xylariaceae were further compared by the phylogenetic analysis out of both rDNA LSU D1/D2 and ITS regions. The sequences of all xylariaceous isolates were aligned using CLUSTAL X v.1.8 (Thompson et al., 1997), and further visual alignments were done in Sequence Alignment Editor (Se-Al) v.2.0 (Rambaut, 2000). The aligned sequences were analyzed together with outgroup species of Neocosmospora vasinfecta (AY381155) in D1/D2 analysis and Pestalotiopsis sp. (AB440094) in ITS analysis. Moreover, 7 species of Xylaria, 2 species of Hypoxylon and 6 species of Xylariaceae from GenBank were added in D1/D2 analysis, and 7 species of Xylaria, 2 species of Hypoxylon and one species of Nemania sp. (EF682111) were added in ITS analysis. These species from GenBank were analyzed with the isolates in this study (Table 2). The aligned sequences were analyzed with the kimura 2-parameter distance matrix by pairwise distances with PAUP*4.0 beta 10 (Swofford, 2002). Bootstrap (Felsenstein, 1985) values were generated with 1,000 replicate heuristic searches to estimate support for clade stability of the consensus tree.
using the same program.

Antimicrobial assay of endophytic fungi

Isolates of endophytic fungi from Lauraceae and Rutaceae were tested for antagonistic effects against plant pathogens using dual-culture method. Primary test showed that four isolates, of endophytic fungi, including Lasmenia sp. (isolate CB10), Ophioceras tenuisporum (isolate CI02), Xylaria cubensis (isolate LA04) and Cyanodermella sp. (isolate TR09), can inhibit the mycelial growth of C. gloeosporioides TC01. These isolates were further tested for the inhibition on nine isolates of plant fungal pathogens and three strains of plant bacterial pathogens by a dual culture method. For the dual culture test, an agar block (0.5 cm in diameter) containing mycelial mates was removed from each endophyte grown on PDA for 7–14 days and inoculated at the center of PDA in a Petri dish (9 cm in diameter). After incubation at 25°C for 7–14 days, each endophyte was tested against plant pathogens by spraying 500 μl of spore suspensions (10^6 spores/ml) of each fungal pathogen or 500 μl of bacterial suspensions (10^7 cfu/ml) of each bacterial pathogen in each Petri dish. After incubation at 25°C for 2 days, the dual culture in each dish was examined for the formation of inhibition zone. The inhibition index is 0 to 3, where 0 = no inhibition zone; 1 = inhibition zone > 0 < 5 mm; 2 = inhibition zone > 5 < 10 mm; and 3 = inhibition zone > 10 mm. The experiment was repeated twice and for each experiment, there were four replicates in each treatment.

Control of anthracnose of Chinese cabbage by culture filtrates of Lasmenia sp. isolate CB10 (Greenhouse Experiment)

Since Lasmenia sp. isolate CB10 showed a strong antagonistic effect against Colletotrichum higginsianum isolate PA01 in vitro test, this isolate was selected for further testing on control of anthracnose of Chinese cabbage (Brassica rapa L.) under greenhouse conditions. Crude filtrates of Lasmenia sp. isolate CB10 were collected from 2-week-old, PDB (potato dextrose broth) cultures and diluted to 10- and 100-fold with sterile distilled water. Two were the methods of inoculations: 1), 24-day-old plants of Chinese cabbage (cv. San Feng) were sprayed with filtrates of Lamienia sp. and then inoculated with conidia suspensions (2x10^6 conidia/ml) of C. higginsianum PA01 from 7-10 days old PDA cultures four days later and 2), 28-day-old plants of Chinese cabbage were inoculated with conidia suspensions of C. higginsianum for 30 minutes and then sprayed with filtrates of Lasmenia sp. Inoculated plants were kept in the greenhouse at 24–26°C. Chinese cabbage plants sprayed with sterile distilled water or the pathogen alone were used as controls. After incubation for 7 days, plants were examined for symptoms of foliar necrosis and scored for severity of anthracnose based on rating of 0-4, where 0 = no visible symptom; 1 = 1%~10% of necrotic area on leaf; 2 = 11%~25% of necrotic area on leaf; 3 = 26%~50% of necrotic area on leaf; 4 = >50% of necrotic area on leaf (Lin, 2001). The disease severity index was calculated by the formula (N: total number of leaves; n0–n4: number of leaves under each disease index).

For each experiment, there were four replicates for each treatment and three plants per replicate. The experiment was repeated twice.

RESULTS

Diversity of fungal endophytes

A total of 156 isolates of endophytic fungi were isolated from 22 species of medicinal plants in Taiwan. Based on morphological characteristics and rDNA LSU D1/D2 and ITS characteristics, 125 isolates were identified as representatives of 30 genera in 19 families, 7 isolates were identified only to family level, 14 isolates were identified as representatives of 5 genera without
Table 2. Number and identification of endophytic fungi isolated from medicinal herbs of Rutaceae and Lauraceae in Taiwan

<table>
<thead>
<tr>
<th>Fungal family</th>
<th>Fungal genus</th>
<th>Plant species (fungal isolate)</th>
<th>Lauraceae</th>
<th>Rutaceae</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amphisphaeriaceae</strong></td>
<td>Discostroma</td>
<td>Citrus medica (CM08)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pestalotiopsis</td>
<td>Cinnamomum kanehirai (CK01), Cin. osmophloeum (CO01, CO08), Litsea cubeba (LC03)</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Apiosporaceae</strong></td>
<td>Arthrinium</td>
<td>Fortunella japonica (FJ05)</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Botryosphaeria</td>
<td>Cinnamomum kanehirai (CK01), Cin. osmophloeum (CO01, CO08), Litsea cubeba (LC03)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Camarosporium</td>
<td>Citrus medica var. sarcodactylis (CB07)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Botryosphaeriaceae</strong></td>
<td>Guignardia</td>
<td>Citrus sp. (CE02-03), Tetradium ruticarpum (TR07), Z. wutaiense (ZW13)</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chaetomiaceae</strong></td>
<td>Chaetomium</td>
<td>Cit. sinensis (CS04)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Diaporthaceae</strong></td>
<td>Diaporthe</td>
<td>Cit. aurantium (CA05), Todalia asiatica (TA02)</td>
<td>5</td>
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<td></td>
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<tr>
<td></td>
<td>Eutypella</td>
<td>Cit. medica var. sarcodactylis (CB03)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lasiosphaeriaceae</strong></td>
<td>Podospora</td>
<td>Machilus thumbergii (MT08-09, MT11)</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Leptosphaeriaceae</strong></td>
<td>Leptosphaeria</td>
<td>C. kanehirai (CK13)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Halosphaeriaceae</strong></td>
<td>Lanspora</td>
<td>Cinnamomum sp. (WLC02)</td>
<td>4</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Fusarium</td>
<td>Cit. aurantium (CA05), Todalia asiatica (TA04), Z. nitidum (ZN03)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hypocreaceae</strong></td>
<td>Magnafusarium</td>
<td>Citrustaphyllum (FC01)</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myrothecium</td>
<td>Cit. aurantium (CA08-09)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Magnaporthaceae</strong></td>
<td>Mycosphaerella</td>
<td>Todalia asiatica (TA01), Z. nitidum (ZN04)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phomopsis</td>
<td>Cit. aurantium (CA03), F. japonica (FJ03, FJ08)</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Melanconidaceae</strong></td>
<td>Pilidiella</td>
<td>Cit. medica (CM05)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mycosphaerellaceae</strong></td>
<td>Cercospora</td>
<td>Citrus medica var. sarcodactylis (CB01), Citrus sp. (NS01-02), P. amurense (PA02), Todalia asiatica (TA03), Te. ruticarpum (TR03, TR06), Z. wutaiense (ZW02, ZW04)</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pseudocercospora</td>
<td>Cit. sinensis (CS01-02), Cit. aurantium (CA01, CA10), Cit. medica var. sarcodactylis (CB06), P. amurense (PA04), Z. wutaiense (ZW03, ZW05, ZW09)</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phyllachoraceae</strong></td>
<td>Colletotrichum</td>
<td>Citrus medica var. sarcodactylis (CB01), Citrus sp. (NS01-02), P. amurense (PA02), Todalia asiatica (TA03), Te. ruticarpum (TR03, TR06), Z. wutaiense (ZW02, ZW04)</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pleosporaceae</strong></td>
<td>Alternaria</td>
<td>Cit. aurantium (CA04-05), Cit. medica (CM01-04), Cit. sinensis (CS02), Todalia asiatica (TA05), Z. nitidum (ZN01), Z. wutaiense (ZW08, ZW11, ZW25)</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Psathyrellaceae</strong></td>
<td>Coprinopsis</td>
<td>Cit. medica var. sarcodactylis (CB01), Citrus sp. (NS01-02), P. amurense (PA02), Todalia asiatica (TA03), Te. ruticarpum (TR03, TR06), Z. wutaiense (ZW02, ZW04)</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stictidaceae</strong></td>
<td>Cyanoderma</td>
<td>Cit. medica var. sarcodactylis (CB01), Citrus sp. (NS01-02), P. amurense (PA02), Todalia asiatica (TA03), Te. ruticarpum (TR03, TR06), Z. wutaiense (ZW02, ZW04)</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trichosphaeriaceae</strong></td>
<td>Nigrospora</td>
<td>Cit. aurantium (CA04-05), Cit. medica (CM01-04), Cit. sinensis (CS02), Todalia asiatica (TA05), Z. nitidum (ZN01), Z. wutaiense (ZW08, ZW11, ZW25)</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Valsaceae</td>
<td>Pseudocercospora</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xylariaceae</td>
<td>Xylaria</td>
<td>F. japonica (FJ01), Z. wutaiense (ZW12, ZW17)</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>
Phylogenetic analysis of endophytic Xylariaceae

Although Xylaria and Hypoxylon are the two popular genera in medicinal plants in Taiwan, certain other endophytes of Xylariaceae are still obscure based on the morphology and sequence of nucleotide. According to the results of phylogenetic analyses based on rDNA LSU D1/D2 and ITS regions, the 33 isolates of endophytic fungi belonging to Xylariaceae could be divided into two major molecular groups, 19 isolates for Xylaria and 13 isolates for Hypoxylon (Figs. 1, 2). In the group of Hypoxylon, all isolates except isolate LA01, the diversity was 0–0.1% for rDNA LSU D1/D2 sequences and 0–3.1% for rDNA ITS sequences. In the group of Xylaria, the diversity was 0–5.5% for rDNA LSU D1/D2 sequences and 0–30.6% for rDNA ITS sequences. For D1/D2 analysis, the bootstrap was 99% for the group of Hypoxylon and 97% for the group of Xylaria. For ITS analysis, the bootstrap was 90% for the group of Hypoxylon and 89% for the group of Xylaria. Based on ITS region analysis, the results indicated that the unidentified xylariaceous isolates could be classified as Xylaria. However, certain unidentified xylariaceous isolates still could not be classified by D1/D2. Thus, ITS region could contribute more diversity to classify fungi in Xylariaceae.

**Antimicrobial activity assay**

The four isolates of endophytic fungi, Lasmenia sp. isolate CB10, Ophioceras teniusporum isolate CI02, Xylaria cubensis isolate LA04 and Cynadodermella sp. isolate FTR09, showed different level of antagonistic effects against 9 fungal isolates and 3 bacterial strains of plant pathogens (Table 3). Cynadodermella sp. isolate TR09 from Tetradium ruticarpum was highly antagonistic to the fungal pathogens Cynidocladia lageniformis CL01, Fusarium oxysporum f. sp. lilii Fol-04 and Monilinia fructicola TW01 and the bacterial pathogen Ralstonia solanacearum PW2, with an
Table 3. Antimicrobial activities of endophytic fungi against plant pathogens

<table>
<thead>
<tr>
<th>Plant pathogen</th>
<th>Inhibition index(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CB10(^2)</td>
</tr>
<tr>
<td>--=</td>
<td>==</td>
</tr>
<tr>
<td><strong>Fungus</strong></td>
<td></td>
</tr>
<tr>
<td>Alternaria solani AS01</td>
<td>2</td>
</tr>
<tr>
<td>Botrytis cinerea NBC12</td>
<td>1</td>
</tr>
<tr>
<td>Colletotrichum gloeosporioides TC01</td>
<td>3</td>
</tr>
<tr>
<td>Colletotrichum higginsianum PA01</td>
<td>3</td>
</tr>
<tr>
<td>Cylindrocladiella lageniformis CL01</td>
<td>1</td>
</tr>
<tr>
<td>Fusarium oxysporum f. sp. lilii Fol-04</td>
<td>2</td>
</tr>
<tr>
<td>Monilinia fructicola TW01</td>
<td>3</td>
</tr>
<tr>
<td>Penicillium digitatum DOB02</td>
<td>0</td>
</tr>
<tr>
<td>Pestalotiopsis psidii TG01</td>
<td>2</td>
</tr>
<tr>
<td>Pythium aphanidermatum P374</td>
<td>2</td>
</tr>
<tr>
<td><strong>Bacterium</strong></td>
<td></td>
</tr>
<tr>
<td>Erwinia carotovora Ec4</td>
<td>0</td>
</tr>
<tr>
<td>Xanthomonas campestris Xcc17</td>
<td>2</td>
</tr>
<tr>
<td>Ralstonia solanacearum PW2</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^1\) Inhibition zone index: 0 = No inhibition; 1 = inhibition zone 0.-5 mm; 2 = inhibition zone 5-10 mm; 3 = inhibition zone > 10 mm.
\(^2\) CB10: Lasmenia sp. isolated from Citrus medica var. sarcodactylis; C102 isolate: Ophioceras sp. isolated from Cinnamomum insularimontanum; LA04 isolate: Xylaria sp. isolated from Lindera aggregate; TR09 isolate: Cyanodermella sp. isolated from Tetradium ruticarpum.

Inhibition index of 3 (inhibition zone > 10 mm). Lasmenia sp. isolate CB10 from Citrus medica var. sarcodactylis was highly antagonistic to the fungal pathogen Monilinia fructicola TW01, with an inhibition index of 3; whereas Xylaria cubensis isolate LA04 from Lindera aggregate showed only weak or none antagonistic effects to the nine isolates of fungal pathogens, with the inhibition index of 1 (inhibition zone < 5 mm) or 0 (no inhibition) and none antagonistic effects to the three isolates of bacterial pathogens (Table 3).

Control of anthracnose of Chinese cabbage by culture filtrates of Lasmenia sp. isolate CB10 (Greenhouse experiment)

Spraying Chinese cabbage plants with culture filtrates of Lasmenia sp. isolate CB10, diluted by 10- or 100-fold, significantly reduced disease severity of anthracnose of Chinese cabbage compared to the pathogen-inoculated control (disease severity of 50%). The disease severity was 14.5 and 12.5% for the treatment of culture filtrates diluted to 10 folds and 100 folds at 4 days prior to inoculation of the pathogen, however, the disease severity was 37.5 and 31.3% for the treatment of culture filtrates diluted to 10 folds and 100 folds at 30 min after inoculation of the pathogen. Meanwhile, no symptoms of phytotoxicity on Chinese cabbage plants were observed by the treatment of 10- and 100-fold dilution of the culture filtrates of Lasmenia sp. isolate CB10.

DISCUSSION

Based on morphological studies and molecular analyses, one hundred and forty-six of the 156 isolates of endophytic fungi obtained from medicinal plants of Lauraceae and Rutaceae in Taiwan were assigned to 35 genera in 19 families, with 21 genera from Lauraceae and 27 genera from Rutaceae. The fungal flora of this study showed that fungal isolates identified as Sordariomycetes and Dothideomycetes are most abundant fungal endophytes in tropical or subtropical plants as reported in previous studies (Arnold et al., 2007; Higgins et al., 2007; Rodriguez et al., 2009). In this study, fungal genera of Coprinopsis and Cyanodermella were rarely reported as endophytes in...
Fig 1. A Neighbor-joining tree based on partial LSU rDNA sequences of D1/D2 unknown isolates of the Xylariales (33 isolates) and 14 reference sequences from Genbank. Neocosmospora vasinfecta (AY381155) was used as an outgroup to root the tree. Numbers at branch nodes are bootstrap values, indicating branch support, based on 1000 replication.
Fig 2. A Neighbor-joining tree based on partial ITS rDNA sequences unknown isolates of the Xylariales (33 isolates) and 9 reference sequences from Genbank. *Pestalotiopsis* sp. (AB440094) was used as an outgroup to root the tree. Numbers at branch nodes are bootstrap values, indicating branch support, based on 1000 replication.

- 0.01 substitutions/site
Lauraceae and Rutaceae but they were found to occur on these hosts. The new record of Coprinopsis isolated from Citrus medica belongs to Agaricomycetes of basidiomycetes and the Cyanodermella isolated from Zanthoxylum wutaense belongs to Lecanoromycetes of ascomycetes. In addition, Citrus and Zanthoxylum are popular hosts of endophytic fungi in this study. The species of Citrus and Zanthoxylum are popular medicinal herbs, especially the endemic species of Z. wutaense often used by aborigine as medicine in Taiwan. Previous studies indicated that the genera of Alternaria, Cladosporium, Colletotrichum, Guignardia, Nigrospora, Nodulisporium, Sporormiella and Xylariaceae could be obtained from Citrus spp. (Araujo et al., 2001; Durán et al., 2005) and Aspergillus, Cochliobolus, Colletotrichum, Penicillium, Rhizopus, Schizophyllum and Trichoderma could be obtained from Zanthoxylum spp. (Maysarah, 2010). In our study, more than 15 genera of endophytic fungi were isolated from Citrus spp. and more than 14 genera of endophytic fungi were isolated from Zanthoxylum spp. Thus, the fungal endophytes in Citrus and Zanthoxylum are more divertive than other medicinal herbs. Moreover, the results showed that Citrus and Zanthoxylum are important hosts for fungal endophytes.

The genera Phomopsis and Xylaria were reported as common endophytes (Bayman et al., 1998; Davis et al., 2003; Huang et al., 2008; Joshee et al., 2009; Nalini et al., 2005; Wang et al., 2008). In this study, Phomopsis and Xylaria were also common genera in Lauraceae and Rutaceae in Taiwan; however, the genera of Colletotrichum and Hypoxylon were more common endophytes than Xylaria (Table 2). Results of comparing the dominant endophytes between Lauraceae and Rutaceae showed that Colletotrichum, Guignardia, Nigrospora and Phomopsis are commonly obtained from Rutaceae and Hypoxylon and Xylaria are from Lauraceae. Although the xylariaceous fungi are not associated with host species significantly (Okane et al., 2008), the Lauraceae may be one of the plant families showing highly related to Xylariaceae. Moreover, the 6 species of Lauraceae as hosts of Xylaria and Hypoxylon are growing in the plant collection at Fushan Botanical Garden, Ilan which was located in low elevation and belonged to virgin forests. Previous studies indicated that Xylaria and Hypoxylon are common in tropical hosts (Roger, 1979) and are dominant fungal endophytes in the forest (Bayman et al., 1998). Thus, the Xylaria and Hypoxylon are more common in the natural condition than in artificial condition. Presently, Xylaria and Hypoxylon are considered as common saprophytic genera in wood, and the related information of Xylaria and Hypoxylon is not clear. Several reports indicated that Xylaria and Hypoxylon could produce antimicrobial (Liu et al., 2008; Tomcheck et al., 2010) and antitumor (Healy et al., 2004) compounds or phytotoxins (Abate et al., 1997) or cytotoxins (Dagne et al., 1994). Certain endophytic Neotyphodium in grasses have been demonstrated that it can produce alkaloid and cause strongly toxic effects on vertebrates (Faeth, 2002). In this study, one isolate of Xylaria sp. isolate LA04 showed minor effects on mycelial growth of pathogenic fungi, and the result revealed that metabolites produced by Xylaria sp. isolate LA04 have activity to against microbes. It is necessary to analyze the effects of compound produced by Xylaria sp. isolate LA04 to vertebrates in the future.

Eight fungal genera are identified as endophytes for the first time in this study, including Lanspora, Magnaporthe, Ophioceras, Pilidiella and Lasmenia in Sodariomycetes; Cyanodermella in Lecanoromycetes, Streliziana in Eurotiomycetes and Ochroconis in Ascomycetes. Previous reports indicated that certain genera of fungal endophytes could possibly cause diseases in crops (Chen et al., 2011; Wang et al., 2008) or animal (Rippon 1988). For example, certain species of Magnaporthe (Thongkantha et al., 2009) and Pilidiella (Van Niekerk et al., 2004) have been reported as pathogens plants and a number of Ochroconis species are identified as pathogens of poultry (Rippon, 1988). Therefore, further investigation is warranted to confirm the pathogenicity of the endophytic fungi isolated from medicinal herbs of Lauraceae and Rutaceae in Taiwan. Although the genera Xylaria and Hypoxylon are
common and dominant endophytes in tropical forest (Chen et al., 2011; Davis, 2003; Okane et al., 2008; Joshee et al., 2009), most Xylaria species still could not be identified by rDNA of ITS and D1/D2 LSU regions (Okane et al., 2008). In this study, the unknown species identified as Xylariaceae did not have high sequence homology with known species of Xylaria in GenBank. The molecular phylogenetic analysis demonstrated that the unknown species of Xylariaceae are closely related with the species of Xylaria. Moreover, the isolates of Hypoxylon showed simple topology based on D1/D2 LSU rDNA regions analysis and showed diverse topology based on ITS rDNA region analysis. Although the D1/D2 sequences of Hypoxylon isolates have 97% identity with H. fragiforme (EU715613) and H. perforatum (AB376735) in GenBank, the topology revealed that the isolates of Hypoxylon from FBG in Taiwan are distinct from H. fragiforme (EU715613) and H. perforatum (AB376735). The molecular analysis demonstrated that the isolates of Hypoxylon from FBG are different from other Hypoxylon species in GenBank.

Previous reports indicated that some endophytic fungi can produce metabolites with antimicrobial property (Shu et al., 2005; Xu et al., 2008) and with potential for use as biocontrol agents for control of crop diseases (Chaves et al., 2009; Ezra et al., 2004; Gabler et al., 2010; Mejia et al., 2008; Mercier and Jiménez, 2009; Stewart et al., 1993; Wali et al., 2006) and insects (Omacini et al., 2001; Patterson et al., 1991; Siegel et al., 1990). In this study, three isolates, Lasmenia sp. isolate CB10, O. tenuisporum isolate C102 and Cyanodermella sp. isolate TR09, showed high antagonistic effects on the growth of plant pathogens. Results of the experiment in greenhouse showed that culture filtrates of Lasmenia sp. isolate CB10 significantly reduced the disease severities of Chinese cabbage anthracnose. Therefore, fungal endophytes from medicinal herbs in Taiwan have potential to inhibit the pathogens growth and control crop diseases. Moreover, certain endophytic fungi could produce volatile compounds (Gabler et al., 2010; Mercier and Jiménez, 2009), induce resistance (Chaves et al., 2009; Kunkel and Grewal, 2003) and against stress (Redman et al., 2002; Rodriguez et al., 2008; Scharl and Phillips, 1997). In this study, Lasmenia sp. isolate CB10 has been confirmed to produce metabolites showing activity to against plant pathogens. Moreover, the metabolites seem to induce resistance of Chinese cabbage against anthracnose. The mechanism of induced resistance of host plant is related with many signals triggered by aggressors, like chemicals, metabolites extracted from plants and microbes and microorganisms (Pieterse et al., 2009). The nature of metabolites of CB10 involved in induced disease resistance warrants further studies.

In addition, Cit. medica var. sarcodactylis is a host plant of CB10 and the species of medicinal herb contained the compounds that were active against bacteria and fungi (Dahiya and Kumar, 2008; Matsumoto et al., 2002). Thus, it is also important to analyze chemical nature of metabolites of CB10 that showed activities against plant pathogens.

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樟科與云香科之中草藥植物內生真菌的鑑定與抗菌特性

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摘要: 本研究為鑑定分離自臺灣中部與北部中草藥植物之樟科 (67 株) 與云香科 (89 株) 共 156 株的內生真菌。依形態與分子序列鑑定顯示, 156 株內生真菌可被分成 35 屬 19 科, 以 Colletotrichum、Guignardia、Hypoxylon、Nigrospora、Phomopsis 及 Xylaria 等屬真菌被分離頻率較高, 其中又以 Xylariaceae 中的 Hypoxylon 與 Xylaria 屬真菌被分離比率最高。此外云香科中的 Citrus 與 Zanthoxylum 屬植物和樟科中的 Cinnamomum 屬植物, 則最易分離到內生真菌。進一步利用 D1/D2 與 ITS rDNA 序列分析 Xylariaceae 科內生真菌的分子親緣性, 得知所分離 Xylariaceae 科內生真菌主要可分成 Xylaria 與 Hypoxylon 兩個分子群。測試云香科與樟科所分離內生真菌對植物病原之拮抗性, 顯示 Lasmenia sp. CB10 菌株、Ophioceras tenuisporum CI02 菌株、Xylaria cubensis LA04 菌株及 Cyanodermella sp. TR09 菌株對抑制 12 種植物病原真菌、3 種植物病原細菌及 1 種卵菌表現較佳。於溫室評估 CB10 菌株對降低白菜炭疽病之效果, 顯示該菌株可明顯降低白菜炭疽病的發生, 證明 CB10 菌株具有防治白菜炭疽病之潛力。

關鍵詞: 拮抗能力、內生真菌、中草藥植物、樟科、云香科。