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Endophytic fungi from *Taxus mairei* in Taiwan: first report of *Colletotrichum gloeosporioides* as an endophyte of *Taxus mairei*

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ABSTRACT. Forty endophytic fungal isolates were obtained from symptomless leaf samples of nine *Taxus mairei* trees on Fu-Shan, Taiwan. Identification was based on morphological characters and comparison of rDNA ITS sequences to those in GenBank. *Colletotrichum* and *Fusarium* were the endophytic fungi most frequently isolated from leaves of *T. mairei*. *Colletotrichum gloeosporioides* was newly recorded as an endophyte of *T. mairei*.

Keywords: *Colletotrichum gloeosporioides*; Endophytes; *Fusarium*; *Taxus mairei*.

INTRODUCTION

The term "endophyte" was introduced by De Bary (1866) and was initially applied to any organism found within a plant that causes asymptomatic infections entirely within plant tissues but no symptoms of disease (Wilson, 1995). Endophytic fungi have been examined in conifers (Petrini et al., 1992), including *Pinus* spp. (Sieber et al., 1999), *Taxus* spp. (Fisher and Petrini, 1987), and *Juniperus* spp. (Petrini and Müller, 1979; Petrini and Carroll, 1981), and they are presumed to be ubiquitous.

Endophytic fungi have been described as playing a protective role against insect herbivory not only in grasses (Clay, 1990) but also in conifers (Carroll, 1991). *Taxus mairei* is important due to its production of taxol and the production of taxol by at least some of its endophytes.

Wang et al. (2000) screened 45 endophytic fungi isolated from the inner bark of *T. mairei* and found that *Tubercularia* sp. strain TF5 produced taxol. In another study that examined hundreds of endophytic fungi from *T. mairei*, *Cephalataxus fortunei*, and *Torreya grandis* (Wang et al., 2000), the cytotoxin brefeldin A was found to be produced by *Paecilomyces* sp. and *Aspergillus clavatus*. Caruso et al. (2000) screened 150 fungal strains isolated from *Taxus baccata* and *Taxus brevifolia*, 15 strains produced taxanes. Huang et al. (2001) screened 172 endophyte isolates from *T. mairei*, *C. fortunei*, and *T. grandis* to analyze the antitumor and antifungal activities.

Most previous reports on the endophytic fungi of *Taxus mairei* focus on their ability to produce important anticancer agents, such as taxol. In the present study, we investigate the diversity of the endophytic fungi in *T. mairei* from Taiwan.

MATERIALS AND METHODS

Plant materials and fungal isolates

Thirteen symptomless leaf samples were randomly collected from nine *T. mairei* trees from Fu-Shan Nature Reserve, Ilan (24°34' N, 121°34' E, 750 m elevation), Taiwan. Samples were collected and processed within 24 h after collection.

The method of sterilization was modified from Guo et al. (2001). The leaves were surface-sterilized by soaking for 3 min in a solution of 1% sodium hypochlorite and then for 30 s in sterile water. Specimens were cut aseptically into 5-mm-long segments, blotted dry on sterile paper towels, and placed onto 2% water agar. Cultures were incubated at room temperature (20-24°C) and microscopically observed daily for the emergence of fungal mycelium and to check whether hyphae grew from the inner tissue, not on the outside. After 1-3 days, the inner hyphal tips were removed and transferred to potato dextrose agar. Initial identification of these isolates was achieved based on conidial and colony morphology by some identification keys. Different morphological isolates were identified, grouped, and then randomly selected for DNA analyses.

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DNA extraction, PCR, and sequencing

DNA from mycelium was extracted by the CTAB method (Doyle and Doyle, 1990). The PCR amplification of the rDNA ITS region was undertaken using the universal ITS5 and ITS4 primers (White et al., 1990). PCR products were purified using minicolumns (Wizard PCR Preps DNA purification System, Promega) according to the manufacturer's protocol and directly sequenced in ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). Primers ITS5 and ITS4 were used in the bi-directional sequencing reaction, which was repeated at least thrice to confirm the sequence data. Using BLAST, we searched GenBank for sequences that were similar to those isolated in our study. The most similar reference sequences were recorded.

RESULTS

Leaf samples of the nine *T. mairei* trees yielded 40 endophytic fungal isolates. They did not produce sexual reproductive structures in cultures. Based on a microscopic observation of mycelia and conidia, these endophytes were identified as *Colletotrichum gloeosporioides*, *Fusarium solani*, *Aspergillus nidulan*, and several isolates identified by genus, including *Colletotrichum* sp., *Phanerochaete* sp. and *Rhizoctonia* sp. Besides these, three species had sterile mycelia only.

The rDNA internal transcribed spacer (ITS) region sequences are now used in the identification and detection of fungi (Pryor and Gilbertson, 2000; Anderson et al., 2001; Guo et al., 2001). ITS sequences of about 600 bp were obtained for 17 isolates representing nine taxa identified by morphological characters. Using the program FASTA, the GenBank and EMBL databases were screened for ITS sequences of fungal taxa that closely matched ours. Eleven reference sequences of nine fungal species were obtained (Table 1).

In Fu-Shan Nature Reserve, nine species of endophytic fungi in five genera and three unidentified fungal endophytes were isolated from nine *T. mairei* trees (Table 1). The most frequently isolated genera were *Colletotrichum* and *Fusarium*. *Colletotrichum gloeosporioides* was isolated from leaf samples of seven trees, and thus the isolation frequency was 77.8%. The isolation frequency of the *Fusarium solani* was 66.7%.

Colletotrichum gloeosporioides and a *Fusarium solani* were both present in five leaf samples of *T. mairei* collected from Fu-Shan. In fact, one sample had four endophytes species, *Colletotrichum* sp., *Fusarium* sp., and two unidentified endophytes, sp.1 and sp.3, which demonstrates the diversity of fungal endophytes in *T. mairei* leaves.

Tm1-5 and Tm6-3-2 were *Fusarium solani* and Tm-6 was *Aspergillus nidulans*. They all showed at least a 98% similarity to reference sequences (Table 1). Tm1-1 and Tm1-4 were *Phanerochaete sordida*, and *Rhizoctonia solani*, respectively. They showed a 97% similarity to reference sequences (Table 1).

Endophyte sp.1 Tm4-6 showed a 96% similarity to the reference EF4200019 fungal endophyte. Endophyte sp.2 (Tm3-5 and Tm8-1) and sp.3 were Basidiomycetes. They were identical at the ITS region and showed at least a 98% similarity to reference sequences.

DISCUSSION

We surveyed the endophytic fungi diversity of *T. mairei* leaves. We used conidia and mycelia morphology

Table 1. Fungal endophytic isolates recovered from 9 *Taxus mairei* on Fu-Shan, Taiwan. GenBank Accession numbers of the rDNA ITS sequence of isolates used in this study and the similarity with their most closely related fungal ITS sequences in GenBank.

Endophytic isolates in this study					GenBank sequences		
Endophyte taxa	Isolate no.	Tree no.	Isolate code	Accession no.	Blast result	Accession no.	Similarity
Colletotrichum sp.	1	1	Tm3-2	AY452985	*Colletotrichum* sp.	AJ301939	100%
Colletotrichum sp.	1	1	Tm6-1	AY423480	*C. gloeosporioides*	AJ301974	98%
C. gloeosporioides	10	6	Tm4-1	AY423474	*C. gloeosporioides*	AJ301907	99%
C. gloeosporioides	6	4	Tm5-1[1]	AY423475	*C. gloeosporioides*	AJ301986	100%
Fusarium solani	9	6	Tm1-5[2]	AY433806	*Fusarium* sp.	AM412637	99%
Aspergillus nidulans	3	1	Tm6	AY452983	*Aspergillus nidulans*	AF455505	98%
Phanerochaete sp.	1	1	Tm1-1	AY433811	*Phanerochaete sordida*	AB210078	97%
Rhizoctonia solani	1	1	Tm1-4	AY433813	*Rhizoctonia solani*	AF153780	97%
Fungal endophyte sp.1	3	3	Tm4-6	AY433808	Fungal endophyte	EF420019	96%
Fungal endophyte sp.2	3	1	Tm3-5[3]	AY456192	Aphyllophorales	EF060457	98%
Fungal endophyte sp.3	2	2	Tm3-3	AY433812	Basidiomycete	AY730555	99%

[1] Represented 5 isolates, rDNA ITS sequences AY423475 to AY423479.

[2] Represented isolate Tm6-3-2, AY433805.

[3] Represented isolate Tm8-1, AY433810.

to identify the endophytes, and then confirmed the identification by ITS rDNA sequences analysis. Because many fungi of the class Ascomycetes and Basidiomycetes do not produce sexual structures in artificial media, it is difficult to identify these fungi using traditional microscopic methods only.

We limited the present investigation to culturable fungi. In other words, we neglected obligate biotrophs. The biodiversity could thus be underestimated.

Colletotrichum species were the endophytes most frequently isolated from *T. mairei* in Fu-Shan, and these have not yet been reported as endophytes of *Taxus* though they have been reported as common endophytes from other plants (Fröhlich et al., 2000; Larran et al., 2001; Photita et al., 2001; Cannon and Simmons, 2002; Arnold et al., 2003).

It is interesting that anthracnose caused by *C. gloeosporioides* on *T. mairei* has been reported in Taiwan (Fu et al., 2003). This pathogen has appeared on cuttings and seedlings in nurseries and on larger plants grown in plantations. The vigor of both the host and the fungus depends on temperature. Higher temperature places stress on the host plants. Under lower temperature, the host plants are more vigorous and less susceptible to attack. Symptoms appeared within 7 days when the temperature was over 32°C, and symptoms were delayed when it was below 24°C. The average yearly air temperature of Fu-Shan Nature Reserve is 18.3°C (Taiwan Forestry Research Institute Website, http://www.tfri.gov.tw/tfe/2fnew/10.htm). Thus, perhaps due to temperature limitation, *C. gloeosporioides* infects *T. mairei* as a common endophyte without disease expression in Fu-Shan. However, this may merely indicate that some isolates are well adapted for an endophytic mode of life and might be potential pathogens.

The ecological roles of endophytes are diverse and varied. *Colletotrichum gloeosporioides* is a worldwide plant pathogen that infects many plant species (Brown et al., 1998; Jeger and Bailey, 1992). *Colletotrichum musae* and *C. gloeosporioides* have been found as endophytes in banana, but these fungi also cause anthracnose of banana fruits (Photita et al., 2001), supporting the idea that pathogens may spend part of their lives in an endophytic stage (Brown et al., 1998).

Xylariaceous fungi and *Alternaria* were reported as the most commonly isolated endophytes. Other genera commonly isolated include *Idriella*, *Phoma*, *Phomopsis*, and *Phyllosticta* (Rodrigues and Petrini, 1997; Caruso et al., 2000). In this study, *Colletotrichum* species were the most common endophyte in *T. mairei*, but none of the other fungi mentioned above were found.

Fusarium solani endophytic isolates were taken from six trees. ITS sequence comparisons revealed their identities to be: *Cycas taiwaniana*, *Pinus morrisonicola*, *Pinus taiwanensis*, *Pseudotsuga wilsoniana*, *Keteleeria davidiana*, *Taiwania cryptomerioides*, *Chamaecyparis formosensis*, *Taxus baccata*, *Taxus floridana*, and *Calocedrus formosana* from Fu-Shan (data not shown). Thus, *Fusarium solani* was the second most frequently isolated species in our samples.

In previous studies, the genus *Fusarium* is a frequent endophyte in other plants (Bills and Polishook, 1992; Suryanaryanan and Kumaresan, 2000; Photita et al., 2001; Cao et al., 2002; Larran et al., 2002). However, these studies generally do not supply the species name. GenBank contains the ITS sequences of only two *Fusarium* endophytes, *Fusarium arthrosporioides* AY575714 and *Fusarium oxysporum* strain F35 AY555719, and they differ from our ITS sequence of *Fusarium solani*. Our ITS sequence showed 99% similarity (533 bp/536 bp) to the reference sequence AM412637, *Fusarium solani* (Azor et al., 2007).

The other endophytic species *Aspergillus nidulans* and *Rhizoctonia solani* have been described in previous studies (Bills and Polishook, 1992; Rodrigues and Petrini, 1997; Suryanaryanan and Kumaresan, 2000; Photita et al., 2001). The results obtained in this work are in agreement with Wang et al. (2002), who reported *Aspergillus* sp. as a common endophytic fungus in their investigation of *T. mairei*.

Phanerochaete and *Rhizoctonia* are both common saprobic fungi. *Phanerochaete* causes white rot of conifer logs and stumps. When the host is weakened, it is the dominant fungus of the decaying process (Eriksson et al., 1990). Petrini (1991) mentioned the symptomless tissues may be in ecological species equilibrium, and when senescence process starts, this tissue gradually allows the establishment of new, mainly saprobic fungal species.

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台灣紅豆杉的內生真菌：
首次報告膠孢炭疽菌為紅豆杉的內生菌

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本研究由福山九株紅豆杉的無病徵葉片分離出 40 株內生真菌，以形態特徵與比對核醣體核酸內間隔轉錄區序列鑑定這些內生真菌。炭疽菌和鐮胞菌是紅豆杉葉片上最常被分到的內生真菌，其中膠孢炭疽菌首次報導為紅豆杉的內生菌。

關鍵詞：內生真菌；紅豆杉；膠孢炭疽菌；鐮胞菌。