

# Phylogenetic Diversity and Antifungal Activity of Endophytic Fungi Associated with *Tephrosia purpurea*

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**Abstract** Sixty-one endophytic fungus strains with different colony morphologies were isolated from the leaves, stems and roots of *Tephrosia purpurea* with colonization rates of 66.95%, 37.50%, and 26.92%, respectively. Based on internal transcribed spacer sequence analysis, 61 isolates were classified into 16 genera belonging to 3 classes under the phylum Ascomycota. Of the 61 isolates, 6 (9.84%) exhibited antifungal activity against one or more indicator plant pathogenic fungi according to the dual culture test. Isolate TPL25 had the broadest antifungal spectrum of activity, and isolate TPL35 was active against 5 plant pathogenic fungi. Furthermore, culture filtrates of TPL25 and TPL35 exhibited greater than 80% growth inhibition against *Sclerotinia sclerotiorum*. We conclude that the endophytic fungal strains TPL25 and TPL35 are promising sources of bioactive compounds.

**Keywords** Antifungal activity, Endophytic fungi, ITS sequence, *Tephrosia purpurea*

Currently, endophytic fungi are of biotechnological interest due to their potential use as biological control agents and as sources of novel, biologically active secondary metabolites. Many of the bioactive agents that are produced by plants (e.g., taxol) can also be produced by endophytic fungi [1]. Endophytic fungi yield a broad variety of substances, including antioxidants, novel antibiotics, and antimicrobial, immunosuppressant and antiparasitic compounds, and thus are rich sources of biologically active metabolites that have been widely exploited in medicine, agriculture, and industry [2, 3]. Here, we isolated endophytic fungi from

the plant *Tephrosia purpurea*.

*T. purpurea* is widely distributed in tropical, sub-tropical and arid regions. This perennial herb is an ingredient in traditional herbal formulations for hepatitis [4] and exhibits several hepatoprotective activities, including antimicrobial [5], wound-healing [6], antiulcer [7], immunomodulatory [8], and anticancer [9] activities. The plant is used commonly as an anti-inflammatory agent in the traditional Indian system of medicine [4]. Several phytochemicals have been isolated from *T. purpurea*, and their medicinal uses have been examined. Previous studies have mainly focused on the phytochemical and pharmacological activities of the plant. However, the role of the endophytic fungi that are associated with *T. purpurea* remains unclear.

To search for bioactive metabolites from endophytic fungi, we collected the medicinal plant *T. purpurea* which was not reported to be attacked by many plant pathogens or pests and then isolated the endophytic fungi. Here, we investigated the phylogenetic diversity of endophytic fungi from *T. purpurea* and evaluated their potential as biocontrol agents against a variety of pathogenic fungi. To our knowledge, this work is the first report describing endophytic fungi from *T. purpurea*.

## MATERIALS AND METHODS

**Sample collection.** Healthy and asymptomatic leaves, stems and roots were collected randomly from 8 *T. purpurea*

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plants in the campus of Hunan Agricultural University, Changsha, China, from June to October 2013. Each tissue sample was used within 48 hr of collection. Finally, the plant parts were washed with running tap water to remove attached soil and then rinsed twice with distilled water and processed to isolate the endophytic fungi.

**Surface sterilization and isolation of endophytic fungi.** To kill epiphytic microorganisms, the samples were initially surface sterilized according to Petrini *et al.* [10], with some modifications. Samples from each tissue were immersed in 75% ethanol for 3–5 min soaked in a 0.1% mercuric chloride solution for 30–45 sec, depending on the tissue, and finally rinsed five times with distilled water. The samples were then dried on sterile tissue paper and cut into small pieces using a sterile pinch cutter. The leaves were cut into 0.5 × 0.5-cm squares, and the stems and roots were cut into 0.5-cm segments. Then, three to five segments were placed onto potato dextrose agar (PDA) containing 0.5 g/L streptomycin. All plates were incubated in the dark at 26°C, and observations were recorded daily for 4 wk. The hyphal tips of the developing fungal colonies were transferred to fresh PDA plates to obtain pure cultures. All strains were stored in 30% glycerol in a deep freezer at –80°C.

The colonization rate was calculated as the total number of segments colonized by endophytic fungi divided by the total number of incubated segments [11].

#### Phylogenetic analysis of culturable endophytic fungi.

Actively growing mycelium of the endophytic fungi was scraped directly from the PDA plates. Genomic DNA was extracted using a fungal genomic DNA extraction kit (Sangon, Shanghai, China). The sequences for the consensus fungal primer ITS4 and ITS5 regions were 5′-TCCTCCG-CTTATTGATATGC-3′ and 5′-GGAAGTAAAAGTCGTA-

ACAAGG-3′, respectively [12]. DNA was amplified in a final volume of 50 µL containing 1 µL of template DNA, 2 µL of 10 pmol of each primer, and 25 µL of Dream Taq Green PCR Master Mix (2×) (Thermo Scientific, Waltham, MA, USA). The cycling program used was as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 56°C for 30 sec, and 72°C for 45 sec; finally, the reaction was incubated at 72°C for 10 min. The resulting PCR products were resolved using electrophoresis on 1% agarose gel in 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1 mM ethylenediaminetetraacetic acid pH 8.0) running buffer and then stained with ethidium bromide and photographed under UV light. Sequencing was performed by a commercial company (Sangon).

The sequences of the internal transcribed spacer fragments (ITS4–ITS5) were analyzed using the nucleotide BLAST program; the National Center for Biotechnology Information (NCBI) database was used to test for similarity. Phylogenetic trees were constructed using the neighbor-joining method of MEGA ver. 5 [13] including a bootstrap analysis of 1,000 replications. The confirmed rDNA sequences for each species were then deposited in GenBank under the accession numbers listed in Table 1.

#### Bioassay of endophytic fungi against plant pathogenic fungi.

Cultures of the isolates were screened for their ability to interact with the *Sclerotinia sclerotiorum*, *Phytophthora parasitica* var. *nicotianae*, *Phytophthora melonis*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and *Rhizoctonia solani*, plant pathogens of global importance that cause significant yield loss across many crops. Postharvest phytopathogenic fungi were obtained from the culture collection of the Plant Pathology Laboratory, Plant Protection Department, Hunan Agricultural University, Changsha, China. For dual culture testing, 6-mm disks containing endophytic fungi and plant pathogenic fungi

**Table 1.** The codes and GenBank accession numbers of the isolated strains

Strains	GenBank accession No.	Strains	GenBank accession No.	Strains	GenBank accession No.
TPL01	KJ849280	TPL13_TPS21	KJ863509	TPR05_TPR06_TPL18 <sup>a</sup>	KJ863523
TPL09_TPL21 <sup>b</sup>	KJ863496	TPL15	KJ863510	TPR07	KJ863524
TPS20	KJ863497	TPL40_TPL45	KJ863511	TPR09_TPL31	KJ863525
TPL07	KJ863498	TPL22	KJ863512	TPR12	KJ863526
TPL17	KJ863499	TPL28	KJ863513	TPS01	KJ863527
TPS03	KJ863500	TPL35_TPL44	KJ863514	TPS06	KJ863528
TPL03	KJ863501	TPL37	KJ863515	TPS08	KJ863529
TPL04	KJ863502	TPL42	KJ863516	TPS09	KJ863530
TPL05_TPL06	KJ863503	TPL46	KJ863517	TPL51_TPL32_TPL39	KJ863531
TPL08_TPL30	KJ863504	TPL50_TPL14	KJ863518	TPL43_TPS12	KJ863532
TPL10	KJ863505	TPR01_TPL23	KJ863519	TPS16_TPL34	KJ863533
TPL41	KJ863506	TPL31	KJ863520	TPS17	KJ863534
TPL24	KJ863507	TPR08_TPR02	KJ863521	TPS23_TPS14_TPL47	KJ863535
TPL11	KJ863508	TPR04	KJ863522	TPL25	KP663722

<sup>a</sup>Three strains.

<sup>b</sup>Two strains: Although these strains exhibited different culture characteristics on potato dextrose agar, they had the same base site; thus, only one sequence was submitted to GenBank.

were placed 4 cm apart on PDA. The plates were incubated at 26°C for 3~7 days in the dark. The width of the inhibition zones between the pathogen and the endophytic fungi was scored as antifungal activity and was measured in millimeters. The result was recorded as no inhibition (-), weak inhibition (+), moderate inhibition (++) and strong inhibition (+++).

The culture filtrates of strains exhibiting strong activity were tested using the poisoned food technique of Grover and Moore [14] against six plant pathogenic fungi. Two plugs of mycelial agar, 6 mm in diameter, were obtained from the growing edges of 7-day-old cultures of endophytic fungus strains and cultured in a potato dextrose broth (PDB) medium (100 mL per conical flask) for 5~7 days at 26°C and 200 rpm. Four flasks containing PDB medium were inoculated with each endophytic fungus, and one flask of medium was maintained as a control. The cultures were then centrifuged to remove hyphal mass at 4,000 rpm for 15 min, and the supernatant containing the excretory metabolites of the endophytic fungus was then filter-sterilized through a Millex-GP 0.22- $\mu$ m syringe filter (Millipore, Billerica, MA, USA) before assaying the antifungal activity. PDA (15 mL) was poured into sterilized 75-mm Petri dishes, and sterilized culture filtrate (1 mL) of endophytic fungus was then added. The medium was supplemented with the same amount of PDB medium instead of culture filtrate for the control sets. Upon solidification of the medium, plant pathogen was inoculated at the center of the plate, and growth inhibition of the treatment against the control was measured by mycelial growth inhibition and calculated according to the formula of Pandey *et al.* [15]:

$$\text{Percentage of mycelial growth inhibition} = (dc - dt)/dc \times 100,$$

where  $dc$  = the average diameter (in mm) of fungal colonies in the control, and  $dt$  = the average diameter (in mm) of fungal colonies in the treatment groups.

## RESULTS

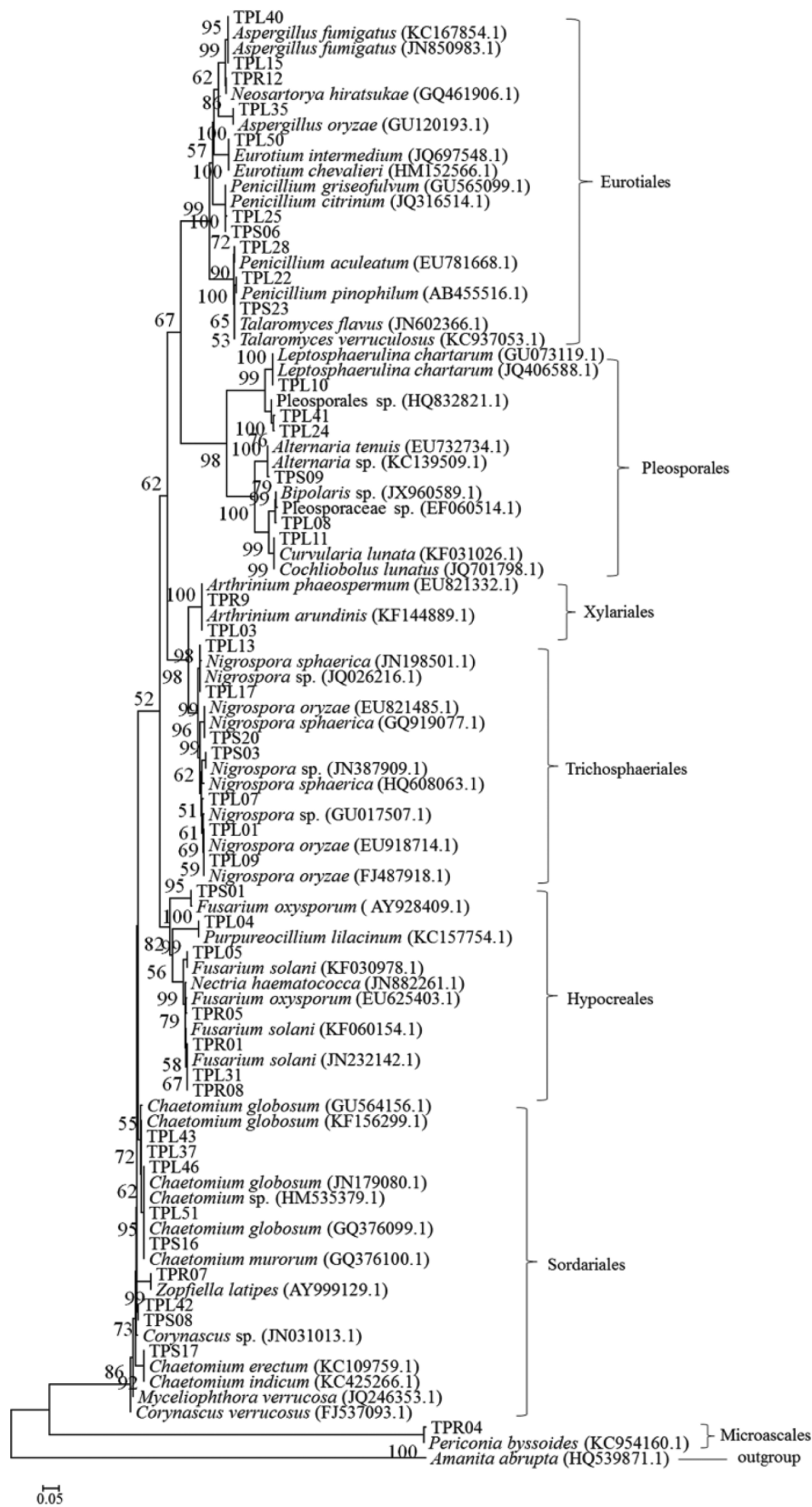
**Diversity of culturable endophytic fungi associated with *T. purpurea*.** Sixty-one endophytic fungi with different colony morphologies among 239 isolated were isolated from 440 tissue segments (236 leaves, 152 stems, and 52 roots) of *T. purpurea*. Little difference was found in the colonization rates among the leaf (66.95%), stem (37.50%) and root (26.92%) samples (Table 2). Thirty-nine isolates originated from leaf samples, 13 were isolated from stems, and 9 were isolated from roots (Table 3). These endophytic fungi were identified by rDNA sequencing of the internal transcribed spacer region and by phylogenetic analysis. All isolated strains belonged to one of 3 classes—Sordariomycetes, Dothideomycetes and Eurotiomycetes—including the following 7 orders: Sordariales, Microascales, Hypocreales, Xylariales, Trichosphaeriales, Eurotiales, and Pleosporales. Of the 61 strains, 59 were identified at the genus level, including *Chaetomium*, *Zopfiella*, *Fusarium*, *Purpureocillium*, *Arthrimum*, *Nigrospora*, *Eurotium*, *Aspergillus*, *Penicillium*, *Neosartorya*, *Talaromyces*, *Alternaria*, *Curvularia*, *Leptosphaerulina*, *Bipolaris*, and *Periconia*, and 2 strains

**Table 2.** Colonization rates of endophytic fungi on leaves, stems and roots of *Tephrosia purpurea*

	Leaves	Stems	Roots	Overall
No. of samples	236	152	52	440
No. of isolates recovered	158	57	14	229
Colonization rate (%)	66.95	37.50	26.92	52.05

**Table 3.** Distribution of endophytic fungi in *Tephrosia purpurea*

Order	Family	Genus	No. of isolates			
			Total	Leaves	Stems	Roots
Sordariales	Chaetomiaceae	<i>Chaetomium</i>	12	9	4	-
		<i>Zopfiella</i>	1	-	-	1
Microascales	Halosphaeriaceae	<i>Periconia</i>	1	-	-	1
Hypocreales	Nectriaceae	<i>Fusarium</i>	11	5	1	5
	Ophiocordycipitaceae	<i>Purpureocillium</i>	1	1	-	-
Xylariales	Apiosporaceae	<i>Arthrimum</i>	3	1	1	1
Trichosphaeriales	Trichosphaeriaceae	<i>Nigrospora</i>	9	6	3	-
Eurotiales	Aspergillaceae	<i>Eurotium</i>	2	2	-	-
		<i>Aspergillus</i>	5	5	-	-
		<i>Penicillium</i>	6	4	2	-
		<i>Neosartorya</i>	1	-	-	1
		<i>Talaromyces</i>	1	-	1	-
Pleosporales	Pleosporaceae	<i>Alternaria</i>	1	-	1	-
		<i>Curvularia</i>	2	2	-	-
		<i>Bipolaris</i>	1	1	-	-
		<i>Leptosphaerulina</i>	1	1	-	-
Total			61	39	13	9



**Fig. 1.** Phylogenetic tree of identified isolates that are associated with *Tephrosia purpurea*. The numbers at the nodes are percentages that indicate the levels of bootstrap support from 1,000 pseudoreplicates based on a neighbor-joining analysis. Bootstrap support values greater than 50% are shown above each branch. The scale bar represents 0.05 substitutions per nucleotide position.

were identified at the order level, indicating that a great diversity of taxa are associated with *T. purpurea*. The phylogenetic relationships between these isolates and their related fungi are shown in Fig. 1. All of the isolated endophytic fungi were members of phylum Ascomycota.

**Antifungal activity of the endophytic fungi.** The antifungal activity of endophytic fungi isolated from *T. purpurea* measured by dual culture testing is shown in Table 4. Most of the fungi did not exhibit antifungal activity against six pathogenic fungi; however, 6 isolates did exhibit activity. In this experiment, the isolate TPL25 exhibited the broadest antifungal activity spectrum (Table 4); this isolate strongly inhibited the growth of *S. sclerotiorum* (Fig. 2) and *P. melonis*. TPL35 and TPL04 exhibited strong growth inhibition of *S. sclerotiorum* (Fig. 2) and exhibited high activity against *P. parasitica* var. *nicotianae* and *P. melonis*.

Three isolates (TPR04, TPL15, and TPR05) exhibited moderate to weak antifungal activity against 3 or 4 species of pathogenic fungi of plants. Based on comparative efficacy, among the 6 endophytic fungi isolated from *T. purpurea*, TPL25 and TPL35 exhibited the strongest antifungal potential.

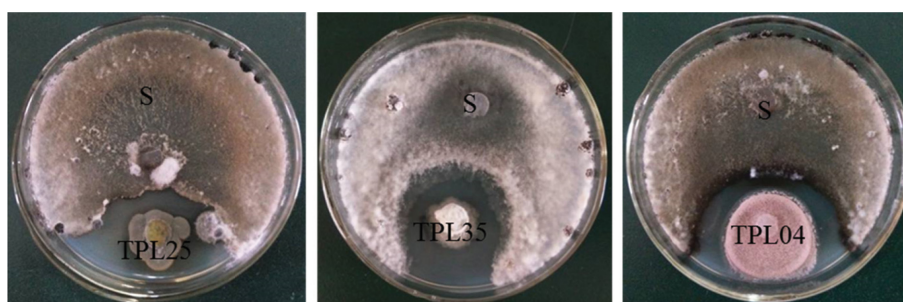
**Bioactivity of endophytic fungi culture filtrates against pathogenic fungi.** Culture filtrates of the endophytic fungi TPL25 and TPL35, which exhibited the strongest antifungal activity in dual culture testing, were tested using the poisoned food technique against six species of pathogenic fungi. As shown in Table 5 and Fig. 3, the culture filtrate of TPL25 exhibited a broad range of antifungal activity against all tested plant pathogens, including *S. sclerotiorum* (85.29%), *P. parasitica* var. *nicotianae* (83.82%), *P. melonis* (82.35%), *R. solani* (40.69%), *B. cinerea* (35.33%), and *C.*

**Table 4.** Identification of endophytic fungi and activity against pathogenic fungi of plants according to the dual culture technique

Strain	Distinguishing morphological characteristics on PDA	Identified as	Potential antifungal activity <sup>a</sup>					
			A <sup>b</sup>	B	C	D	E	F
TPL25	Conidiophores irregular ter- or quarter-verticillate, with branches strongly divergent; Phialides were more or less cylindrical with a very short neck, usually less than 6.5 µm	<i>Penicillium griseofulvum</i>	+++	++	+++	++	++	++
TPL35	Colonies, floccose in olive buffy colors, reverse pale brown; metulae or phialides covering most of the surface of the vesicle	<i>Aspergillus oryzae</i>	+++	-	++	++	+	+++
TPL04	Colonies, white at first, becoming vinaceous; conidia in divergent chains, fusiform; stalks with roughened thick walls; phialides 6~9 × 2.5~3 µm	<i>Purpureocillium lilacinum</i>	+++	-	+++	++	++	-
TPR04	Colonies, becoming pale to dark brown with age, reverse dark brown; mycelium cottony, white, soon forming dark clusters of conidia scattered on the surface; conidiophores with a stipe and swollen apex	<i>Periconia</i> sp.	-	+	+	+	-	++
TPL15	Conidia globose, greyish turquoise, 2~3 µm in diameter; exudate yellowish; reverse yellowish brown; phialides curving to be roughly parallel to each other	<i>Aspergillus fumigatus</i>	+	-	++	-	++	+
TPR05	Colonies, covered with cottony and aerial hyphae, white to pink with aging; falcate conidia formed abundantly	<i>Fusarium</i> sp.	++	++	-	+	-	-

<sup>a</sup>Width of growth inhibition zone (T): -, T = 0 mm; +, 0 < T ≤ 2 mm; ++, 2 < T ≤ 5 mm; +++, T > 5 mm.

<sup>b</sup>A, *Sclerotinia sclerotiorum*; B, *Rhizoctonia solani*; C, *Phytophthora melonis*; D, *Colletotrichum gloeosporioides*; E, *Botrytis cinerea*; F, *Phytophthora parasitica* var. *nicotianae*.



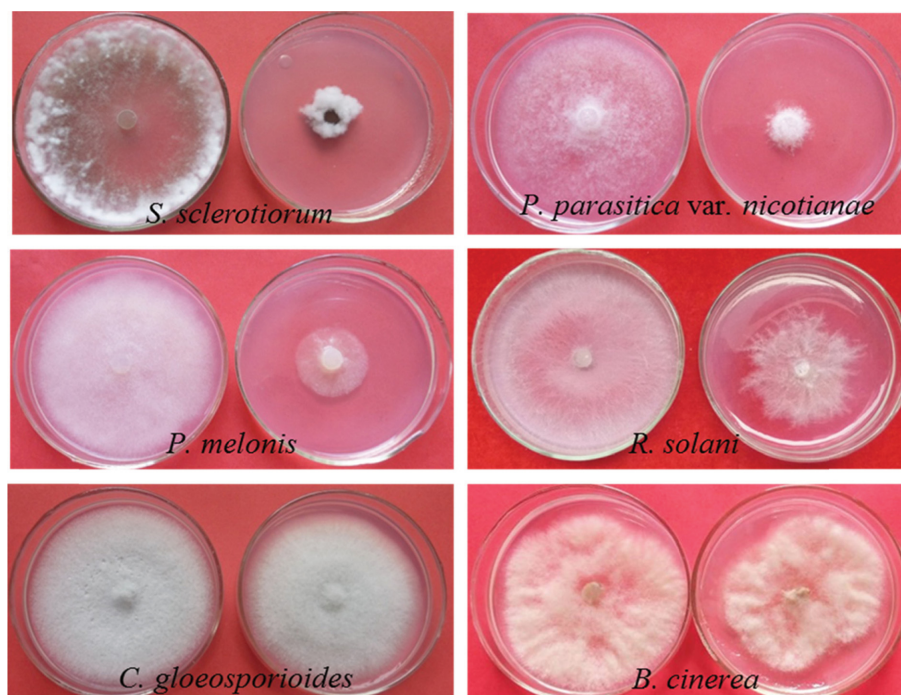
**Fig. 2.** Endophytic fungi from *Tephrosia purpurea* showing activity in a dual culture antagonistic study against the fungal pathogen *Sclerotinia sclerotiorum* (S).

**Table 5.** Activity of culture filtrates of endophytic fungi against pathogenic fungi of plants

Fungus No.	Growth inhibition (%) <sup>a</sup>					
	A <sup>b</sup>	B	C	D	E	F
TPL25	85.29 ± 7.13	40.69 ± 6.32	82.35 ± 2.82	14.71 ± 1.40	35.33 ± 11.18	83.82 ± 3.28
TPL35	84.31 ± 0.85	0.00 ± 0.00	34.80 ± 5.57	8.00 ± 2.77	48.61 ± 5.76	60.29 ± 16.25

<sup>a</sup>Each value represents the mean of three replicates ± standard deviation.

<sup>b</sup>A, *Sclerotinia sclerotiorum*; B, *Rhizoctonia solani*; C, *Phytophthora melonis*; D, *Colletotrichum gloeosporioides*; E, *Botrytis cinerea*; F, *Phytophthora parasitica* var. *nicotianae*.



**Fig. 3.** Growth inhibition of pathogenic fungus mycelia when treated with the TPL25 culture filtrate. The left plate in each picture was the control, and the right plate was treated with the TPL25 culture filtrate. *S. sclerotiorum*, *Sclerotinia sclerotiorum*; *R. solani*, *Rhizoctonia solani*; *P. melonis*, *Phytophthora melonis*; *C. gloeosporioides*, *Colletotrichum gloeosporioides*; *B. cinerea*, *Botrytis cinerea*; *P. parasitica* var. *nicotianae*, *Phytophthora parasitica* var. *nicotianae*.

*gloeosporioides* (14.71%). Among the tested plant pathogens, *S. sclerotiorum* (84.31%) and *P. parasitica* var. *nicotianae* (60.29%) were inhibited most by the culture filtrate of TPL35; however, the culture filtrate of TPL35 exhibited moderate antifungal effects against *B. cinerea* (48.61%), *P. melonis* (34.80%) and *C. gloeosporioides* (8.00%), and did not exhibit antifungal activity against *R. solani*.

## DISCUSSION

Previous studies have noted that medicinal plants represent a potent, economically important source of microbial diversity [16-18]. Therefore, *T. purpurea* was used as a source to screen for antimicrobial endophytic fungi. In this study, endophytic fungi were successfully found in the leaves, stems and roots of this plant. Endophytic fungi were more prevalent on leaf tissues (66.95%) than on the stem (37.50%) or in root tissues (26.92%), similar to the

results found in previous studies, in which endophytic fungi were isolated most often from leaves and stems, followed by the roots, with average colonization rates of 74.2, 55.6% and 9.4%, respectively [19]. This result was only slightly different from that obtained with *Stipa grandis* (which presented significantly higher endophytic fungi colonization rates in roots than in leaves) [20]. Interestingly, the endophytic fungi distribution patterns differed between the leaves, stems and roots. This phenomenon might be affected by tissue texture and differences in the tissue physiology and chemistry [21]. Compared with the above-ground parts, the root tissues of *T. purpurea* yielded an almost completely different endophytic mycoflora, which was characterized by low isolation rates and a different species composition.

In this study, *Chaetomium* and *Fusarium* species were the dominant endophytic fungal species in *T. purpurea*. *Chaetomium* species have often been reported to be

endophytic fungi in host plants including *Huperzia serrata* [22], *Nyctanthes arbor-tristis* [23], *Cinnamomum camphora* [24] and *Lycopersicon esculentum* [25]. It has also been reported that the metabolites of *Chaetomium* sp. have bioactivity, including antifungal [26], antioxidant [27] and anticancer [28] activities. *Fusarium* is a cosmopolitan and common plant pathogen in nature, particularly *Fusarium solani*, which causes root rot resulting in considerable losses in many important crops [29]. However, *Fusarium solani* has also been reported as an endophytic fungi on the root tissues of tomato plants [30] and is present as an endophyte in *Apodytes dimidiata*, where it produces camptothecin and 10-hydroxycamptothecin, important precursors for the synthesis of the clinically useful anticancer drugs topotecan and irinotecan [31].

In contrast, only one strain was isolated from the roots of *T. purpurea*, and this strain belonged to the genus *Periconia* under the order Microascales, which might be of practical value. Li *et al.* [32] used *Periconia* sp., endophytic fungus isolated from *Torreya grandifolia*, to produce readily detectable quantities of the anticancer drug taxol. In another study, *Periconia* sp. (obtained from *Piper longum* L.) were used to produce piperine (5-(3,4-methylenedioxyphenyl)-1-piperidinopent-2,4-dien-1-one) under liquid culture [33]. Several interesting biologically active metabolites obtained from the endophytic fungi of medicinal plants have been studied [34]; for example, the polyketide compound 5-hydroxyramulosin, obtained from an endophytic *Phoma* sp. of *Cinnamomum mollissimum*, inhibited the fungal pathogen *Aspergillus niger* [35]. In our study, dual culture assay and culture filtrate testing were employed to evaluate the antimicrobial activity of sixty-one *T. purpurea* strains against pathogenic fungi of plants. Of the isolated strains, only 9.84% were active against one or more indicator pathogenic fungi used in the primary screening, supporting the viewpoints of Gong and Guo [36] and Zhao *et al.* [37]. Thus, the isolates obtained appear to exhibit narrow antimicrobial spectra, with some exceptions [38]. Ding *et al.* [38] found that fermentation broths of most of the endophytic fungi isolates they isolated from *Camptotheca acuminata* exhibited antifungal activity. It was suggested that the percentage of antibiotic-producing strains depends on the assay method used and on the species and the number of indicator microbes used in the screening [39]. The preliminary bioassay presented here might provide further guidance for the screening and identification of novel antimicrobial agents from this potential host plant.

In the dual culture assay, isolate TPL25 (identified as *Penicillium griseofulvum*) exhibited the broadest antifungal activity spectrum, and isolate TPL35 (identified as *Aspergillus oryzae*) was active against 5 fungal plant pathogens. *Penicillium griseofulvum* is well known for its production of griseofulvin, a widely useful antifungal antibiotic metabolic product that was isolated by Oxford *et al.* [40]. Kwon *et al.* [41] isolated two new potent compounds, phenylpropene A and B, from a fermentation broth of *P. griseofulvum* F1959, which

inhibit Acyl-CoA : cholesterol acyltransferase. This represents the first report of the isolation of *P. griseofulvum* as an endophytic fungus from a perennial herb. *Aspergillus oryzae* is important for the production of traditional fermented foods and beverages in Japan [42]. Ali *et al.* [43] reported that *A. oryzae* yields high amounts of L-dopa, which is a useful drug for Parkinson's disease. Furthermore, *A. oryzae* was also reported to exhibit antitumor activity in the study by Zhou *et al.* [44]. The activity of *A. oryzae* against pathogenic fungi, such as *S. sclerotiorum* and *P. parasitica* var. *nicotianae*, is reported here for the first time.

Mycelial growth inhibition assay of the strains TPL25 and TPL35 corroborated the activity exhibited by their fermentation broths. In addition, the results of the bioassays conducted here (in which greater than 80% growth inhibition was observed against some pathogenic fungi of plants) can be considered promising because they were obtained with culture filtrates that were not subjected to purification or concentration; the fermentation of endophytic fungi offers several advantages for producing bioactive compounds, including reproducibility and dependable production. Further research is warranted to identify the active metabolites in the culture filtrates and to evaluate these compounds as possible antimicrobials.

To the best of our knowledge, this study is the first ever collection of, and phylogenetic diversity analysis of, endophytic fungi from *T. purpurea*. The antifungal activities found provide a strong foundation for the isolation and purification of natural antimicrobial agents from the endophytic fungi of *T. purpurea*; these agents might prove useful in designing novel drugs that would provide sustainable solutions to various problems faced by modern society.

## ACKNOWLEDGEMENTS

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