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Diversity and communities of culturable endophytic fungi from the root holoparasite *Balanophora polyandra* Griff. and their antibacterial and antioxidant activities

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Abstract

Purpose: *Balanophora polyandra* Griff. is a holoparasitic medicinal plant that produces compounds with antibacterial and antioxidant activities. Plant endophytic fungi are an abundant reservoir of bioactive metabolites for medicinal exploitation, and an increasing number of novel bioactive compounds are being isolated from endophytic fungi. The present study investigated the diversity of culturable endophytic fungi from the roots of holoparasite *B. polyandra* to explore active strains and metabolites. In addition, the antibacterial and antioxidant activities of 22 strains cultured from *B. polyandra* were also evaluated.

Methods: The endophytic fungi were identified according to their colony morphology and ITS-5.8S rDNA sequencing. TLC-MTT-Bioautography assays and DPPH radical scavenging assays were employed to assess the antibacterial and antioxidant activities of ethyl acetate extracts of the endophytic fungi.

Results: One hundred and twenty-five endophytic strains were isolated from the roots of *B. polyandra*, including 70 from female samples and 55 from male samples. Of them, twenty-two distinct isolates representing 15 genera and 22 species based on their ITS-rDNA genomic sequence were successfully identified from female and male samples of *B. polyandra*. The genus *Calonectria* was the most prevalent genus, with a CF% of 18.3, followed by the genera *Clonostachys* and *Botryosphaeria*, with CF% values of 13.4 and 10.0, respectively. Interestingly, the fungal extracts exhibited broad-spectrum antibacterial activities against gram-positive and gram-negative bacteria, as well as potential antioxidant activities with IC_{50} values ranging from 0.45 to 6.90 mg/mL. Among them, endophytes Bpf-10 (*Diaporthe* sp.) and Bpf-11 (*Botryosphaeria* sp.) showed the strongest biological activities and more abundant secondary metabolites.

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Conclusions: This study reported the diversity of endophytic fungi from the roots of *B. polyandra* and the antibacterial and antioxidant activities of the crude extracts for the first time. The results revealed that *B. polyandra* contains diverse culturable endophytic fungi that potentially produce natural antibacterial and antioxidant compounds with great value to the agriculture and pharmaceutical industries.

Keywords: *Balanophora polyandra* Griff., Endophytic fungi, Secondary metabolites, Antibacterial activity, Antioxidant activity

Background

Plant endophytic fungi are microorganisms that grow inside plant tissues without causing negative symptoms to the host and produce biologically active substances (Sheik and Chandrashekar 2018). They have been regarded as a novel source of natural bioactive compounds with tremendous applications in medicine, agriculture, and the food industry. In the past few years, many valuable bioactive compounds with anticancer, insecticidal, antimicrobial, and cytotoxic activities have been successfully isolated from endophytic fungi (Ascêncio et al. 2014; Jia et al. 2016; Atiphasaworn et al. 2017; Bedi et al. 2017). Endophytic fungi produce bioactive compounds similar to the host plant (Venieraki et al. 2017). Thus, endophytic fungi can be used to isolate active metabolites and reduce the large-scale utilization of plants as a method to protect the environment.

Balanophora polyandra Griff., belonging to the family Balanophoraceae, is a natural medicinal parasitic plant that lives in the root system of many Fagaceae plants and is mainly distributed in southern China, Japan, Nepal, India, and Burma (Wang et al. 2006; Tao et al. 2009). The whole plant has been used as a folk medicine due to its antipyretic, antidotal, and hemostatic properties. Moreover, *B. polyandra* has also been used as a traditional Chinese medicine, especially for treating gonorrhea, syphilis, wounds, and bleeding of the alimentary tract (Wang et al. 2013). Previous studies have shown the presence of potentially active metabolites in *B. polyandra* with antioxidant, immunosuppressive, hypoglycemic, antitumor, and antibacterial activities (Wang et al. 2013; Ouyang et al. 2017). However, no reports have discussed endophytic fungi and their biological activities. Therefore, the diversity of endophytic fungi of *B. polyandra* with biological effects must be elucidated.

The threat of drug-resistant pathogens has become a major concern worldwide (Nafis et al. 2018). *Ralstonia solanacearum* is a soil-borne bacterium that causes bacterial wilt in eucalyptus plantations worldwide, and efficient control measures are still limited (Mao et al. 2021). Similarly, the oxidative stress caused by free radicals is known to be involved in pathophysiological events, especially in some human diseases, such as diabetes mellitus, aging, atherosclerosis, Alzheimer's disease, and Parkinson's disease (Gunasekaran et al. 2017). The main characteristic of antioxidant compounds is capturing and stabilizing free radicals. The development of effective and safe drugs to combat human, animal, and plant diseases is now urgently needed (Patil et al. 2016). One approach to solving these problems is to search for new antibacterial and antioxidant metabolites. Endophytic fungi broaden the scope of new antibiotics, chemotherapeutic agents, and agrochemicals with high efficiency and low toxicity (Hateet 2016; Das et al. 2017; Zhong et al. 2017).

A promising future for developing a new drug exists by exploiting and utilizing endophytic fungi resources from medicinal plants. Hence, this study aims to screen potential endophytic fungi with significant antibacterial and antioxidant activities from the roots of *B. polyandra.* Molecular and morphological approaches were used for the isolation, characterization, and analysis of the diversity of endophytic fungi. Furthermore, the antibacterial and antioxidant activities of endophytic fungal extracts were assessed using TLC-MTT-Bioautography assays and DPPH radical scavenging assays. Finally, the chemical compositions of the crude extracts that had significant biological activities were analyzed using HPLC.

Results

Isolation, identification, and phylogenetic analysis of endophytic fungi isolated from *B. polyandra*

A total of 125 endophytic fungi were isolated from 60 samples of *B. polyandra* (30 female samples and 30 male samples). Seventy fungal isolates were obtained from female samples, and fifty-five were obtained from male samples. According to their colony morphology (shape of conidia, mycelial growth rate, colony color and texture, etc.), 22 distinct fungal isolates (Bpf-1~Bpf-22) were selected for further molecular and microscopic identification. The colonies of Bpf-1~Bpf-22 grown on PDA medium are shown in Fig. 1. The obtained ITS sequences were compared with those in GenBank to identify the fungi. They were identified as members of fifteen genera, including *Clonostachys* (Bpf-1 and Bpf-15), *Gliocladiopsis* (Bpf-2), *Calonectria* (Bpf-3, Bpf-5, Bpf-9, Bpf-15 and Bpf-18), *Gliocephalotrichum* (Bpf-4), *Pestalotiopsis* (Bpf-6), *Botryosphaeria* (Bpf-7 and Bpf-11), *Trichoderma* (Bpf-8), *Diaporthe* (Bpf-10), *Myrothecium* (Bpf-12),

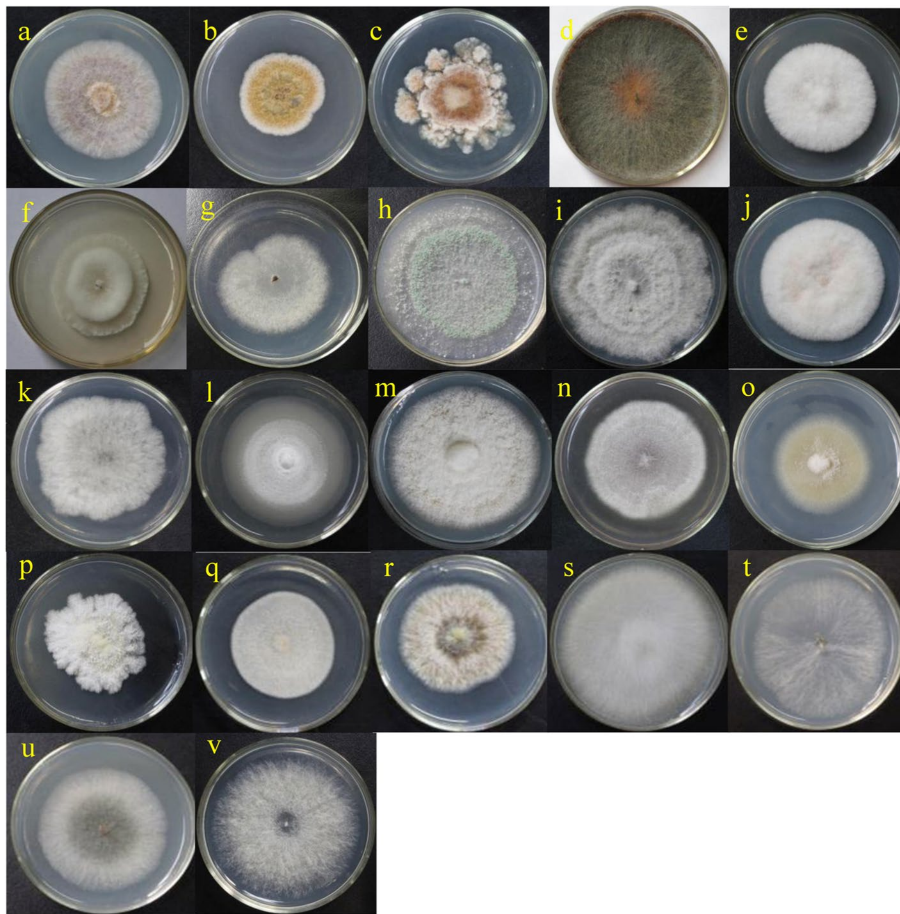

Fig. 1 Front views of the colonies of endophytic fungi isolated from *B. polyandra*. **a**~**v** Bpf-1 to Bpf-22, respectively

Cylindrocladium (Bpf-13), *Fusarium* (Bpf-14 and Bpf-16), *Colletotrichum* (Bpf-17 and Bpf-20), *Mucor* (Bpf-19), *Lasiodiplodia* (Bpf-20), and *Neofusicoccum* (Bpf-22) (Table 1). *Calonectria* was the most prevalent genus, with a colonization frequency (CF) of 18.3%, followed by *Clonostachys* and *Botryosphaeria* with CF values of 13.4% and 10.0%, respectively. The genetic identities of 22 isolates were greater than 98%. The obtained ITS sequences of 22 isolates were submitted to GenBank to obtain their accession numbers (MH378888 and MH378889; MH397479-MH397498) and the closest related species were identified from a BLASTn analysis. The identified fungi with their accession numbers, the closest related species, the percentage of identity, and colonization frequency are presented in Table 1.

Assessment of endophytic fungal diversity

The detailed results of the analysis of endophytic fungal diversity in male and female samples associated with *B. polyandra* are listed in Table 2. Larger values indicated the richness of the endophytic fungi in the samples. According to the Margalef abundance index (D'), female samples (2.354) had a higher value than male samples (2.246), showing the richness of the endophytic fungi in female samples compared to male samples. Moreover, Simpson's (D) and Shannon's (H') diversity indices in female samples were relatively higher than those in male samples ($D = 0.881$ and 0.869; $H' = 2.247$ and 2.126, respectively), suggesting that these endophytic fungi preferentially colonize female samples. However, the Pielou species evenness index (J) of female samples (0.923) was similar to that of male samples (0.937), indicating a uniform species composition across both hosts. The values of D', D, H', and J in the whole tissue were 2.900, 0.898, 2.469, and 0.912, respectively. These values indicate the higher diversity of endophytic fungi in *B. polyandra*.

Antibacterial activity of the endophytic fungal extracts

The antibacterial activity of the fungal extracts against five test bacteria (*Escherichia coli*, *Pseudomonas lachrymans*, *Xanthomonas vesicatoria*, *Ralstonia solanacearum* and *Bacillus subtilis*) are summarized in Table 3. All 22

Table 1 Colonization frequency (CF) of the endophytic fungi isolated from *B. polyandra* and their closest relatives based on the data from the BLASTn analysis

Fungal isolate	Accession number	Closest related species	Identity (%)	Macro-and microscopic identification	CF (%)	Female	Male
Bpf-1	MH378888	KR812215.1 *Clonostachys rogersoniana*	98.78	*Clonostachys* sp.	11.6	+	+
Bpf-2	MH378889	KX274071.1 *Gliocladiopsis forsbergii*	99.83	*Gliocladiopsis* sp.	3.3	+	+
Bpf-3	MH397479	JF742647.1 *Calonectria colhounii*	99.82	*Calonectria* sp.	1.6	+	+
Bpf-4	MH397480	KU203324.1 *Gliocephalotrichum* sp.	100.00	*Gliocephalotrichum* sp.	5.0	+	+
Bpf-5	MH397481	GQ280568.1 *Calonectria curvispora*	99.83	*Calonectria* sp.	8.3	+	+
Bpf-6	MH397482	GU592005.1 *Pestalotiopsis* sp.	100.00	*Pestalotiopsis* sp.	1.6	+	+
Bpf-7	MH397483	KC492452.1 *Botryosphaeria dothidea*	99.35	*Botryosphaeria* sp.	5.0	+	−
Bpf-8	MH397484	KF856960.1 *Trichoderma hamatum*	99.68	*Trichoderma* sp.	3.3	+	−
Bpf-9	MH397485	GQ280594.1 *Calonectria malesiana*	99.65	*Calonectria* sp.	6.6	+	+
Bpf-10	MH397486	KC145898.1 *Diaporthe neotheicola*	98.48	*Diaporthe* sp.	6.6	+	−
Bpf-11	MH397487	MG273743.1 *Botryosphaeria dothidea*	99.44	*Botryosphaeria* sp.	5.0	+	−
Bpf-12	MH397488	KT022226.1 *Myrothecium* sp.	100.00	*Myrothecium* sp.	1.6	+	−
Bpf-13	MH397489	KU896169.1 *Cylindrocladiella lageniformis*	99.46	*Cylindrocladiella* sp.	3.3	+	−
Bpf-14	MH397490	MF076622.1 *Fusarium solani*	98.65	*Fusarium* sp.	3.3	+	+
Bpf-15	MH397491	KY378958.1 *Clonostachys rosea*	99.15	*Clonostachys* sp.	1.6	+	−
Bpf-16	MH397492	KT313628.1 *Fusarium* sp.	98.48	*Fusarium* sp.	3.3	+	−
Bpf-17	MH397493	AJ301932.1 *Colletotrichum acutatum*	100.00	*Colletotrichum* sp.	1.6	−	+
Bpf-18	MH397494	JN794044.1 *Calonectria pseudoreteaudii*	98.78	*Calonectria* sp.	1.6	−	+
Bpf-19	MH397495	KP676592.1 *Mucor irregularis*	99.83	*Mucor* sp.	5.0	−	+
Bpf-20	MH397496	FJ904834.1 *Lasiodiplodia pseudotheobromae*	100.00	*Lasiodiplodia* sp.	6.6	−	+
Bpf-21	MH397497	KT390195.1 *Colletotrichum gloeosporioides*	100.00	*Colletotrichum* sp.	6.6	−	+
Bpf-22	MH397498	HQ832811.1 *Neofusicoccum sp.*	100.00	*Neofusicoccum* sp.	6.6	−	+

+, endophytic fungus were isolated from female samples or male samples; −, endophytic fungus were not isolated from female samples or male samples

endophytic fungal extracts showed antibacterial activity against all the test bacteria to different degrees. For instance, the extracts of endophytes Bpf-1, Bpf-3, Bpf-4, Bpf-8, Bpf-9, Bpf-10, Bpf-11, Bpf-12, Bpf-14, and Bpf-22 were more active as antibacterial agents than the extracts of other endophytes. Among them, Bpf-1, Bpf-11, and Bpf-14 showed the highest inhibitory activity against all the test bacteria with inhibition zone diameters exceeding 10 mm. On the other hand, some fungal extracts (i.e., Bpf-7, Bpf-15, and Bpf-19) also exhibited inhibitory activity against all five test bacteria, and the inhibition zone diameters mainly ranged from 5 mm to 10 mm. The extracts of three endophytes (Bpf-5, Bpf-6, and Bpf-13) showed comparatively weak or no inhibition against all test bacteria. Among the test bacteria, *E. coli* was less susceptible to the endophytic fungal extracts, except for five samples (Bpf-1, Bpf-10, Bpf-11, Bpf-12 and Bpf-14), while *R. solanacearum* and *X. vesicatoria* were generally more susceptible.

The magnitude of the R_f value allowed us to determine the polarity of the compounds separated through our elution system. Based on the results of the antibacterial activity results, the R_f values of extracts exhibiting antibacterial activity all ranged from 0.00 to 0.65. Thus, the secondary metabolites of these endophytic fungi were mainly small to moderately polar substances. In this investigation, the gram-negative bacteria were generally more sensitive to the 22 endophytic fungal extracts than the gram-positive bacteria.

Antioxidant activity of the endophytic fungal extracts

The antioxidant activities of 22 endophytic fungal extracts from *B. polyandra* were evaluated using a DPPH radical scavenging assay. As shown in Fig. 2, all the extracts showed antioxidant activity to varying extents, with IC_{50} values ranging from 0.45 to 6.9 mg/mL. The extracts of endophytes Bpf-10 (*Diaporthe* sp.) and Bpf-11 (*Botryosphaeria* sp.) showed a stronger ability to inhibit DPPH radicals, with IC_{50} values of 0.46 and 0.45 mg/mL, respectively. However, their activities were lower than that of the standard BHT (0.02 mg/mL). The extracts of endophytes Bpf-12, Bpf-13, and Bpf-14 did not show any significant differences, and their IC_{50} values were 0.75, 0.73, and 0.71 mg/mL, respectively. The extract of the endophyte Bpf-21 showed the lowest antioxidant activity, with an IC_{50} value of 6.90 mg/mL.

Table 2 Diversity of endophytic fungi isolated from *B. polyandra*

Taxon	Female	Male	The whole sample
Clonostachys sp.	13	7	20
Gliocladiopsis sp.	4	1	5
Calonectria sp.	14	9	23
Gliocephalotrichum sp.	4	2	6
Pestalotiopsis sp.	1	2	3
Botryosphaeria sp.	14	0	14
Trichoderma sp.	3	0	3
Diaporthe sp.	8	0	8
Myrothecium sp.	1	0	1
Cylindrocladiella sp.	3	0	3
Fusarium sp.	5	3	8
Colletotrichum sp.	0	10	10
Mucor sp.	0	6	6
Lasiodiplodia sp.	0	7	7
Neofusicoccum sp.	0	8	8
Number of total fungal isolates	70	55	125
Margalef's richness index (D')	2.354	2.246	2.900
Simpson's index (D)	0.881	0.869	0.898
Shannon's diversity index (H')	2.247	2.126	2.469
Pielou evenness index (J')	0.937	0.923	0.912

HPLC analysis of the extracts of endophytes with significant activities

Based on the results of strong antibacterial and antioxidant activities, the extracts of endophyte Bpf-10 (*Diaporthe* sp.) and Bpf-11 (*Botryosphaeria* sp.) were selected for further HPLC analysis (Fig. 3). These secondary metabolites mainly had retention times between 2 min and 6 min and between 10 min and 19 min, indicating that endophytes Bpf-10 and Bpf-11 contained major compounds with different polarities. Several fractions suggested that more than one compound produced by two endophytes was responsible for the bioactivity. However, further experiments are required to confirm whether the compounds detected in extracts mediate the antibacterial and antioxidant activities. The obtained HPLC chromatogram provides a theoretical reference for the further isolation, purification and identification of active components from endophytes Bpf-10 and Bpf-11.

Discussion

Endophytic fungi, which are potential producers of medicinal substances, have attracted increasing attention in recent years (Wei et al. 2020). Medicinal plants provide a unique eco-environment for their endophytic fungi. A plethora of previous studies reported that special eco-environmental endophytic fungi might produce special bioactive natural products (Jia et al. 2016). Based on these considerations, we investigated the application of endophytic fungi, especially those isolated from *B. polyandra*, to evaluate their diversity and biological properties.

Endophytic fungi are detected in different medicinal plants worldwide. Their phylogenetic diversity has been reported in various forms to describe the interaction of fungi with the host plant (Tejesvi et al. 2011; Murdiyah 2017). In the present study, 125 endophytic fungi were isolated from *B. polyandra*, and 22 isolates were identified successfully based on morphological features and a sequence analysis of the ITS regions. These isolates showed 98.48–100.00% similarity to their assigned taxa. These fungal isolates belonged to one phylum, three classes, six orders and fifteen genera, showing the phylogenetic diversity of the endophytic fungi. *Calonectria* was the dominant genus and has also been reported in other plants, such as *Acacia persea*, *Sarcococca hookeriana*, and *Buxus sempervirens* (Dann et al. 2012; Wight et al. 2016). Members of a variety of common endophytic genera observed in the present study, such as *Trichoderma*, *Fusarium* and *Colletotrichum*, were typically isolated from different hosts (Hidayat et al. 2016; Ntuba-Jua et al. 2017). In the whole tissue, the large values of H' 2.469 and D' 2.900 revealed that *B. polyandra* hosted rich and diverse endophytic fungi.

Some research groups have reported the antibacterial activity of endophytic fungi from medicinal plants against various pathogenic microbes (Sathish et al. 2014; Liu et al. 2016; Wahab et al. 2017). Another objective of this study was to assess the antibacterial and antioxidant activities of endophytic fungal extracts. All extracts (Bpf-1 to Bpf-22) exhibited broad-spectrum antibacterial activities against *E. coli*, *P. lachrymans*, *X. vesicatoria*, *R. solanacearum*, and *B. subtilis*. Among them, Bpf-10 and Bpf-11 showed the strongest antibacterial activities, suggesting that they could be used as a potential source of antibacterial agents. Our results are supported by previous studies that demonstrated *Diaporthe* LGMF907 had potent antibacterial activity against *E. coli*, *Saccharomyces cerevisiae*, methicillin-sensitive *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (de Medeiros et al. 2018). In addition, *Botryosphaeria* MGN23-3 also displayed strong antibacterial activity against *Bacillus cereus* and *B. subtilis* (da Silva et al. 2022). The remaining fungal extracts showed different activities toward the different tested bacteria, which might result from morphological differences in the cell walls of these pathogens (Gunasekaran et al. 2017). *R. solanacearum* and *X. vesicatoria* were sensitive to most fungal extracts. This result corroborated the findings reported by Ouyang (Ouyang

Table 3 Antibacterial activities of crude extracts of endophytic fungi isolated from *B. polyandra*

Fungal isolate	**R_f values (Inhibition zone diameter)**				
	E. coli	***P. lachrymans***	***X. vesicatoria***	***B. subtilis***	***R. solanacearum***
Bpf-1	0.00–0.35^{++}; 0.38–0.50^{+++}	0.00–0.52^{+++}	0.00–0.52^{+++}	0.00–0.28^{+++}; 0.30–0.38^{++}	0.00–0.58^{+++}
Bpf-2	0.00–0.18^{++}; 0.30–0.34^{+}	0.35–0.38^{+}	0.00–0.17^{+}; 0.30–0.38^{+}	0.05–0.13^{+}	0.00–0.15^{+}
Bpf-3	0.00–0.53^{++}	0.00–0.55^{++}	0.00–0.63^{+++}	0.00–0.50^{+++}	0.00–0.55^{+++}
Bpf-4	0.00–0.48^{++}	0.00–0.57^{+++}	0.00–0.58^{+++}	0.00–0.48^{+++}	0.00–0.53^{+++}
Bpf-5	0.00–0.17^{++}	0.00–0.15^{+}; 0.33–0.47^{++}	0.00–0.13^{+}	–	0.00–0.13^{+}
Bpf-6	0.00–0.15^{+}	–	0.00–0.13^{+}	0.00–0.13^{+}	0.00–0.10^{++}
Bpf-7	0.00–0.22^{++}; 0.25–0.32^{++}	0.00–0.42^{++}	0.00–0.43^{++}	0.00–0.42^{++}	0.00–0.48^{++}
Bpf-8	0.00–0.45^{++}	0.00–0.53^{++}	0.00–0.58^{+++}	0.00–0.5^{++}	0.00–0.55^{+++}
Bpf-9	0.00–0.48^{++}	0.00–0.43^{++}	0.00–0.55^{+++}	0.00–0.47^{++}	0.00–0.22^{++}; 0.28–0.47^{+++}
Bpf-10	0.00–0.53^{+++}	0.00–0.5^{+++}	0.00–0.58^{+++}	0.00–0.52^{+++}	0.00–0.48^{++}
Bpf-11	0.00–0.58^{+++}	0.00–0.52^{+++}	0.00–0.62^{+++}	0.00–0.53^{+++}	0.00–0.50^{+++}
Bpf-12	0.00–0.13^{++}; 0.15–0.28^{++}; 0.32–0.50^{+++}	0.00–0.25^{++}; 0.27–0.43^{+++}	0.00–0.55^{+++}	0.00–0.48^{+++}	0.00–0.42^{++}
Bpf-13	0.00–0.07^{+}	–	0.00–0.18^{+}	–	0.00–0.05^{+}
Bpf-14	0.00–0.55^{+++}	0.00–0.38^{+++}	0.00–0.43^{+++}	0.00–0.58^{+++}	0.00–0.65^{+++}
Bpf-15	0.00–0.48^{++}	0.00–0.15^{++}	0.00–0.33^{++}	0.00–0.15^{++}; 0.23–0.50^{++}	0.00–0.55^{+++}
Bpf-16	0.00–0.25^{++}	0.00–0.25^{++}	0.00–0.27^{+}	0.00–0.23^{+}	0.00–0.28^{++}
Bpf-17	0.00–0.50^{+}; 0.33–0.47^{++}	0.02–0.07^{+}	0.02–0.05^{+}; 0.1–0.15^{+}	0.00–0.07^{+}	0.00–0.12^{+}; 0.17–0.23^{+}
Bpf-18	0.00–0.17^{++}; 0.18–0.55^{++}	–	0.00–0.08^{+}; 0.13–0.18^{+}	0.00–0.07^{++}; 0.38–0.45^{+}	0.00–0.23^{+}; 0.32–0.37^{+}
Bpf-19	0.00–0.42^{++}	0.00–0.45^{++}	0.00–0.55^{++}	0.00–0.45^{++}	0.00–0.47^{++}
Bpf-20	0.00–0.08^{++}; 0.20–0.27^{+}	0.00–0.08^{+}; 0.23–0.30^{+}	0.00–0.20^{++}	0.18–0.33^{++}; 0.42–0.50^{+}	0.00–0.20^{++}; 0.25–0.33^{+}
Bpf-21	0.00–0.13^{++}; 0.22–0.28^{+}	0.00–0.08^{+}; 0.23–0.30^{+}	0.00–0.22^{++}	0.00–0.37^{++}; 0.43–0.52^{+}	0.00–0.23^{+++}
Bpf-22	0.00–0.15^{+}; 0.23–0.30^{++}	0.00–0.17^{++}	0.00–0.21^{++}; 0.28–0.43^{++}	0.00–0.57^{++}	0.00–0.40^{+++}
Streptomycin sulfate	(++)	(++)	(+++)	(++)	(++)

Petroleum ether and acetone (4:1, V/V) were used as the solvent for TLC. The data presented in the table are the R_f values of the antibacterial activity; –, an inhibition zone was not observed; +, the inhibition zone diameter ranged from 0 to 5 mm; ++, the inhibition zone diameter ranged from 5 to 10 mm; +++, the inhibition zone diameter was greater than 10 mm; the positive control streptomycin sulfate was only sampled on the TLC plate

et al. 2017) that extracts of *B. polyandra* showed significant antibacterial activity against *R. solanacearum.*

In the present study, all endophytic fungal extracts showed different levels of antioxidant activity. The extracts of endophytes Bpf-10 (*Diaporthe* sp.) and Bpf-11 (*Botryosphaeria* sp.) showed the best antioxidant activities based on the reduction of DPPH. As a proof of their efficiency, *Diaporthe* sp. MFLUCC16-0682 and *Botryosphaeria* MGN23-3 were reported to have notable antioxidant activities (Tanapichatsakul et al. 2017; da Silva et al. 2022). In addition, altenusin and djalonensone isolated from *Botryosphaeria* sp. had antioxidant activities (Xiao et al. 2014). Many previous studies have proven that some polyphenol, flavonoid and tannin compounds seem to play an important role in reducing peroxidation (Mazandarani et al. 2014; Kada et al. 2017). However, polyphenols, flavonoids, and tannins compounds were found to be absent from fungal extracts (Sharma et al. 2022). Hence, further confirmation is needed to determine whether a positive correlation exists between the extracts and these compounds. In the present study, all activities of fungal extracts were lower than that of the standard butylated hydroxytoluene (BHT). However, negative results do not mean an absence of the bioactive constituents in all fungal extracts, and they may contain other active chemical components that produce a definite physiological action. In this study, two endophytic fungi Bpf-10 (*Diaporthe* sp.) and Bpf-11 (*Botryosphaeria* sp.) were screened for the first time from medicinal plant *B. polyandra*, which showed strong antibacterial and

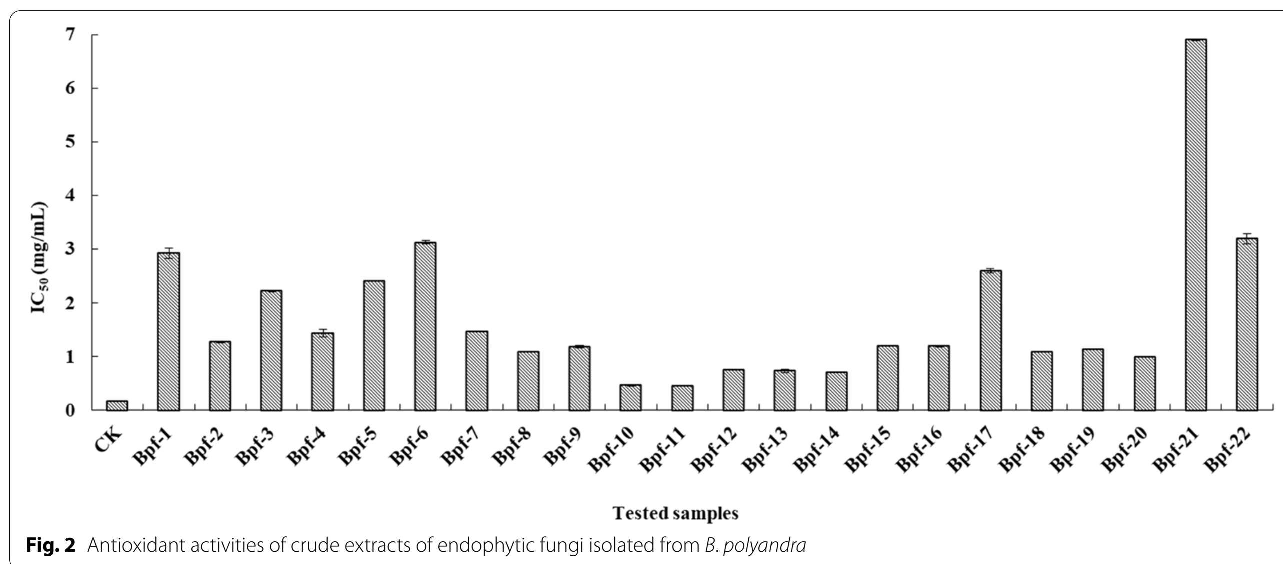

Fig. 2 Antioxidant activities of crude extracts of endophytic fungi isolated from *B. polyandra*

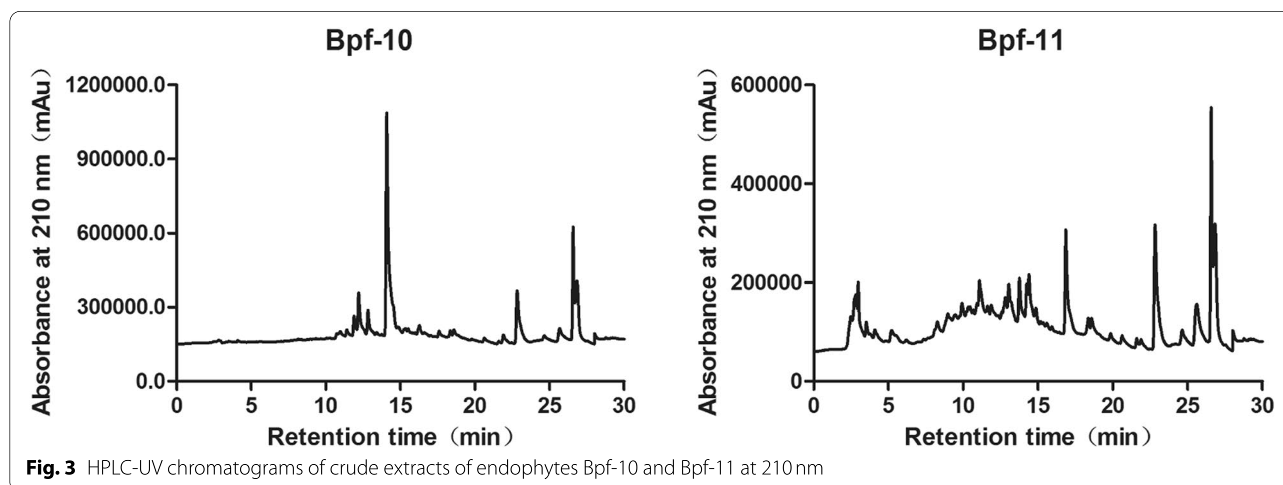

Fig. 3 HPLC-UV chromatograms of crude extracts of endophytes Bpf-10 and Bpf-11 at 210 nm

antioxidant activities. Furthermore, there are few studies on the biological activities of the secondary metabolites of *Gliocladiopsis* (Bpf-2), *Calonectria* (Bpf-3, Bpf-5, Bpf-9, Bpf-15 and Bpf-18), *Gliocephalotrichum* (Bpf-4), and *Cylindrocladium* (Bpf-13).

Conclusions

In this study, the antibacterial and antioxidant activities of ethyl acetate extracts of the culturable endophytic fungi from the root of the holoparasitic plant *B. polyandra* were reported. A total of 125 endophytic fungal isolates were isolated from 60 samples of *B. polyandra* (30 female samples and 30 male samples). Of them, twenty-two distinct isolates (Bpf-1~Bpf-22) were selected for identification and characterization using molecular and morphological analyses. Fifteen genera were identified, among which *Calonectria*, *Clonostachys*, and *Botryosphaeria* were dominant. The crude extracts of *Diaporthe neotheicola* Bpf-10 and *Botryosphaeria dothidea* Bpf-11 showed potent inhibitory activities against DPPH radicals and pathogenic bacteria (*E. coli*, *P. lachrymans*, *X. vesicatoria*, *R. solanacearum*, and *B. subtilis*). Moreover, the HPLC chromatogram showed the presence of secondary metabolites with different polarities in the Bpf-10 and Bpf-11 extracts. These findings indicated that endophytic fungi from the holoparasitic plant *B. polyandra* have great potential to produce antioxidant and antibacterial compounds. Subsequent research will focus on the isolation and identification of the antibacterial and antioxidant compounds from

these fungi, as well as on their applications as biocontrol agents.

Materials and methods

Materials

Whole plants of *B. polyandra* were collected from Chebaling National Nature Reserve, Guangdong Province of China, in September 2015. All the samples were placed in a plastic bag and immediately transported to the laboratory for further study. The plant specimens were authenticated by Dr. Mingxuan Zheng at South China Agricultural University. The plant materials were stored in sealed plastic bags at 4 °C until further use.

Isolation of the endophytic fungi

The endophytic fungi were isolated using the method described by Shan et al. with some modifications (Shan et al. 2019). Plant materials were thoroughly washed for 20 min with running tap water and surface-sterilized with 75% ethanol for 30 s, followed by three rinses with sterilized distilled water. They were then treated with 0.2% mercuric chloride for 20 min and then washed thrice with sterile distilled water. The surface-sterilized tissues were dried on sterile filter papers under aseptic conditions. Finally, each tissue sample was cut into 5 × 5 mm pieces and placed on a potato dextrose agar (PDA) plate containing streptomycin sulfate (500 μg/L). The culture plates were incubated at 28 °C for 1–3 weeks and observed daily. The emerging colonies were subcultured several times on fresh PDA plates to obtain pure isolates. Finally, the pure isolate was transferred into a PDA slant and stored at 4 °C until further use. The colonization frequency (CF %) of each pure isolate was calculated using the following formula:

$$CF = (NCOL/Nt) \times 100\%$$

where "N_{COL}" represents the number of segments colonized by the emerging fungus, and "N_t" represents the total number of samples segment (Shan et al. 2020).

Morphological characterization of endophytic fungi

For morphological identification, all endophytic fungi that varied in shape, growing area, exudate drop color, growth rates, surface texture, reverse color, radial lines, and concentric were observed and identified according to standard taxonomic manuals and textbooks (Praptiwi et al. 2016).

Molecular characterization of endophytic fungi

DNA was extracted from fresh mycelium using a fungal genomic DNA extraction kit (Shanghai Biological Engineering Co., China) according to the manufacturer's method. The ITS region of rDNA was amplified by polymerase chain reaction (PCR) and subsequently sequenced with the universal primers ITS4 (5′-TCCTCC GCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAA AGTCGTAACAAGG-3′). The PCR system was as follows: 25 μL of PCR Master Mix, 21 μL of double-distilled H_2O, 2 μL of template DNA, 1 μL of forward primer ITS4, and 1 μL of reverse primer ITS5. The PCR conditions were set as follows: predenaturation at 95 °C for 2 min; 30 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 40 s, extension at 72 °C for 1 min and 20 s; and extension at 72 °C for 10 min. The PCR products were sequenced and purified by Shanghai Biological Engineering Co., China. The obtained DNA sequences were submitted to GenBank and compared with a BLASTn analysis (Shan et al. 2019).

Assessment of endophytic fungi diversity

The diversity of endophytic fungi at each site was estimated according to Margalef's abundance index (*D'*), Simpson index (*D*), the Shannon-Wiener diversity index (*H'*), and the Pielou species evenness index (*J*). The following formula was used: $D' = (S\text{-}1)/ln\ N$, where "*S*" represents the number of species and "*N*" is the number of individuals in the sample (Cosoveanu et al. 2018). $D = 1 - \Sigma\ Pi^2$, where the ratio "*Pi*" is the frequency of colonization of the taxon in the sample (Zheng et al. 2013). $H' = -\ \Sigma\ Pi\ ln\ Pi$, where "*H'*" was used to show the diversity of the endophytic fungal species (Sadeghi et al. 2019). $J = H'/ln(S)$, where "*J*" denotes the uniformity of the endophytic fungi (Zheng et al. 2013).

Preparation of crude extracts of endophytic fungi

The pure isolates of endophytic fungi were cultivated on PDA plates for 4–7 days. Afterward, 3 to 4 agar plugs with mycelia were inoculated into a 50-mL conical flask containing 20 mL of potato dextrose broth (PDB) (3 flasks for each fungus). All flasks were incubated at 150 rpm on a rotary shaker at 28 °C in the dark for 5–7 days. Each fungal broth and mycelia were inoculated into two flasks containing 20 g of sterile rice for 60 days under aseptic conditions. The fermented product was extracted thrice with ethyl acetate under sonication. Finally, the solvent was evaporated and concentrated using a rotary evaporator to obtain crude extracts. The ethyl acetate crude extracts were stored at 4 °C until use.

Detection of the antibacterial activity of the endophytic fungal extracts

The antibacterial activity of the endophytic fungal extracts was detected using a thin-layer chromatography (TLC)-bioautography assay (Shan et al. 2012). One gram-positive (*B. subtilis*) and four gram-negative (*E. coli*, *P. lachrymans*, *X. vesicatoria*, and *R. solanacearum*)

bacterial strains were used as test bacteria. All the bacterial cultures were reactivated in Luria-Bertani (LB) broth medium for 12 h at 28 °C, followed by streaking on LB agar plates. The bacterial suspension (10^8 CFUs/mL) was mixed with molten semisolid LB medium (with 0.5% agar) before use. Five microliter of the ethyl acetate extracts was spotted onto a silica TLC plate, and then 5 μL of a streptomycin sulfate (CK^+) solution (0.2 mg/mL) was spotted onto the lower right of the TLC plate. The prepared TLC plates were developed in a glass tank with the petroleum ether to acetone (4:1) (v/v) solvent system. The prepared bacterial suspension was poured uniformly over the TLC plate and incubated at 25 °C for 12 h under humid conditions. Last, the TLC plate was sprayed with a 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and then incubated for 2 h. The antibacterial activity of endophytic extracts was assayed by measuring the white inhibition zone diameter on the purple background.

Assessment of the antioxidant activity of the endophytic fungal extracts

The DPPH radical scavenging assay was employed to examine the antioxidant activity of the endophytic fungal extracts using the microtiter plate (96-well) spectrophotometric method (Shan et al. 2019) with some modifications. Briefly, 20 mg of DPPH were dissolved in 100 mL of ethanol to obtain a 0.2 mg/mL DPPH solution (0.004% w/v) for this assay. The stock solutions of test samples were prepared separately by dissolving 0.1 g of ethyl acetate extract in 1 mL of ethanol. A series of stock solutions of different concentrations (20, 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.15625 mg/mL) were prepared by diluting the solutions with ethanol. Butylated hydroxytoluene (BHT) was used as a positive control and prepared using the same method. The final concentrations of BHT were 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125, 0.00625, and 0.003125 mg/mL. After completing the preparation of all solutions, 80 μL of DPPH were added to each well containing 20 μL of sample solutions or BHT solutions at different concentrations. These reaction mixtures were homogenized well and incubated in the dark for 10 min and then incubated for 30 min in a water bath at 37 °C. The absorbance was measured at 517 nm using a spectrophotometer, and tests were performed in triplicate. Ethanol was used as a reference standard. DPPH inhibition was calculated using the following equation: DPPH inhibition (%) = [($A_{517\ nm}$ of control − $A_{517\ nm}$ of sample)/$A_{517\ nm}$ of control] × 100, where A is the absorbance obtained for a sample or the control. The median inhibitory concentration (IC_{50}) value was calculated using a linear relationship between the percentage of DPPH inhibition results and their respective results and predictive equations.

HPLC analysis of the extracts of superior endophytes

High-performance liquid chromatography (HPLC) analysis of the fungal extracts was performed using the gradient elution method (Shan et al. 2020) with an XB-C_{18} reverse-phase (250 mm × 10 mm, 10 μm, Welch, Shanghai, China). Commercial grade water with 0.01% trifluoroacetic acid was used as mobile phase A, and acetonitrile with 0.01% trifluoroacetic acid was used as mobile phase B. The HPLC analysis was performed using a gradient of water to acetonitrile (0–2 min, 20% B, 2–15 min, 20–50% B, 15–16 min 50–100% B, 16–30 min, 100% B) at a flow rate of 1 mL/min, temperature of 40 °C; and UV detection at 210 nm.

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Authors' contributions
W.W, S.W. and T.S. collected the plant material. C.W., W.W, X.W., and T.S. performed the isolation and identification of the endophytic fungi. C.W., W.W, X.W., and T.S. evaluated the antimicrobial activity. C.W., H.S., Y.Y, and Y.W. performed the antioxidant activity. C.W., H.S., and Y.Y contributed in the diversity analysis of endophytic fungi. H.S., Y.Y, and Y.W. performed the HPLC analysis of the crude extract. C.W., W.W, H.S., Y.Y, and T.S. prepared the figures and tables. S.W. and T.S. designed the research. All the authors contributed in writing, editing, and revising the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials
The generated nucleotide sequence of the endophytic fungal isolates (isolation number Bpf-1 ~ Bpf-22) can be accessed in GenBank under accession numbers MH378888, MH378889, MH397479 to MH397488 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The healthy plants of ***B. polyandra*** were collected in September 2015 from Chebaling National Nature Reserve, Guangdong Province, China. The taxonomic identification of the plant materials was performed by Dr. Mingxuan Zheng of College of Forestry and Landscape Architecture (SCAU), where the voucher specimen (SCAULPMH-1509015) of the plant was deposited. All experiments were approved by the College of Forestry and Landscape Architecture (SCAU) and were strictly evaluated in accordance with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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