Differences and biocontrol potential of haustorial endophytic fungi from Taxillus Chinensis on different host plants

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Abstract

Background

In order to explore the community composition and diversity of the endophytic fungi in *Taxillus chinensis*, samples of the parasites growing on seven different hosts, *Morus alba*, *Prunus salicina*, *Phellodendron chinense*, *Bauhinia purpurea*, *Dalbergia odorifera*, *Diospyros kaki* and *Dimocarpus longan*, were isolated. The strains were identified by their morphological characteristics and their internal transcribed spacer (ITS) sequences were analyzed.

Results

150 different endophytic fungi were isolated from the haustorial roots of the seven hosts, and the total isolation rate was 61.24%. These were found to belong to 1 phylum, 2 classes, 7 orders, 9 families, 11 genera and 8 species. *Pestalotiopsis*, *Neopestalotiopsis* and *Diaporthe* were the dominant genera, accounting for 26.67, 17.33 and 31.33% of the total number of strains, respectively. Diversity and similarity analyses showed that the endophytic fungi isolated from *D. longan* (H'=1.60) had the highest diversity index. The highest richness indexes were found in *M. alba* and *D. odorifera* (both 2.23). The evenness index of *D. longan* was the highest (0.82). The similarity coefficient of *D. odorifera* was the most similar to *D. longan* and *M. alba* (33.33%), while the similarity coefficient of *P. chinense* was the lowest (7.69%) with *M. alba* and *D. odorifera*. Nine strains showed antimicrobial activities. Among them, *Pestalotiopsis sp., N. parvum* and *H. investiens* showed significant antifungal activity against three fungal phytopathogens of medicinal plants. At the same time, the crude extracts from the metabolites of the three endophytic fungi had strong inhibitory effect on the three pathogens. *Pestalotiopsis sp., N. parvum* and *H. investiens* had the strongest inhibitory effects of *S. cucurbitacearum*, with inhibitory rates of 100%, 100% and 81.51%, respectively. Secondly, *N. parvum* had a strong inhibitory effect on *D. glomerata* and *C. cassicola*, with inhibitory rates of 82.35% and 72.80%, respectively.

Conclusions

These results indicate that the species composition and diversity of endophytic fungi in the branches of *T. chinensis* were varied in the different hosts and showed good antimicrobial potential in the control of plant pathogens.

Backgroud

Plant endophytic fungi are an important component of the microbial plant symbionts that live within plant tissues, but the hosts usually remain asymptomatic during either some of their lives or throughout their lifetimes [1–2]. They are found to occur naturally in temperate and tropical rainforests and they have about 300,000 land host plants. Every plant has one or more endophytic fungi. It is estimated that more than 1 million endophytic fungal species exist in nature [3]. Bioactive compounds produced by these endophytic fungi (active compounds which are exclusive to them) are important for improving their adaptability to host plants and can increase their tolerance to biological and abiotic stresses. In addition, these compounds can induce the production of a large number of known and novel bioactive secondary metabolites [4–6].

Endophytic fungi not only have a rich biodiversity, but many of their metabolites have a variety of functions, and these can promote the growth of seedlings and improve their resistance to drought, diseases, predatory insects and non-physiological salt concentrations. The accumulated bioactive ingredients can also increase the medicinal efficacy of the host plants and they have an important role with respect their medicinal value as well as other characteristics [5–11]. Since Stierle et al. first reported that an endophytic fungus isolated from *Taxus breviflia* was able to synthesize paclitaxel, an anticancer substance, the study of these types of fungi in medicinal plants has become a research hotspot[12]. For example, Yang et al. found that there were significant differences in the diversity, community structure and composition of endophytic fungi in geiko leaves, and the differences in community structure increased with increasing age of the fungi present. The dominant fungi in the endophytic fungal community were *Aspergillus*, *Candida* and *Mycosphaerella*[13]. Wang et al. found that 749 strains of endophytic fungi could be isolated from the roots, tubers and leaves of wild Diangou plants, belonging to 41 different genera, respectively[14]. Of these, the diversity of endophytic fungi isolated from the tubers was the highest.

Liu et al. found that the community diversity of endophytic fungi in *Astragalus membranaceus* was low, and *Hemileia* and *Gibberella* were the dominant populations of these fungi[15]. Yang et al. found that *Lophiostoma* was the dominant genus in the community composition of endophytic fungi in the bark of *Eucommia ulmoides* from three habitats[16]. Yuan et al. found that the endophytic fungus, *Gilmaniella* sp. Al12, could promote the accumulation of sesquiterpenoid compounds in *Atractylodes lancea* [17]. The fermentation products of endophytic fungi of *Viscum coloratum* had certain anti-tumor activity properties and could cause inhibition of neuraminidase, decreased proliferation of

smooth muscles and bacteriostasis [18]. Taking wheat (*Triticum aestivum* L.) as an example, endophytic fungus of *Azospirillum* sp., could promote the growth of wheat under drought conditions [19]. Some endophytic fungi produced different bioactive compounds, such as alkaloids, diterpenoids, flavonoids and isoflavones, which were able to increase the resistance of their host plants to biological and abiotic stresses [5–6]. *Neotyphodium* has an endophytic fungus which produces alkaloids that can act as a deterrent to host plants and improve their survival from insect attack [20]. For example, inoculation of some endophytic fungi onto crops increased their resistance to insects and subsequently their yield [21]. In addition, this type of fungi-mediated host plant resistance to pathogens was more likely the result in a direct competition between host plants and the pathogens. Dendrobium can promote the seed germination and growth of *Gastrodia elata* by secreting indole-acetic acid [22].

It has also been reported that some endophytic fungi can promote the growth and fitness of host plants by activating the expression of certain enzymes and genes [23]. *Pirospora* promotes tobacco root growth by stimulating the expression of nitrate reductase and starch degrading enzymes (glucan-water dikinase) [24]. For example, specific conditions determine the range of host plants, which in turn determines the species of endophytic fungi and their spore germination, growth, reproduction and metabolism throughout their life cycles. Similarly, the distribution of some endophytic fungal populations is often limited to specific host plant species (or families) and specific genetic background (genotypes). The results showed that temperature, humidity, soil nutrient levels and other ecological and environmental conditions were important factors that determined the type and quantity of secondary metabolites found in host plants, and this indirectly affected the population structure of the endophytic fungi. For example, under conditions of low average annual sunshine hours and high average annual humidity, host plants produce more nutrients suitable for the endophytic fungi to colonize, multiply and spread [25]. In contrast, only certain types of host species can successfully grow in cold climates with inappropriate respiration rates, oxygen concentrations and pH values. Therefore, only a few specific endophytic fungi can be colonized in these corresponding host plants, resulting in a certain regional specificity of endophytic fungi population structure [26]. We also found that the population structure of endophytic fungi is usually region-specific. The distribution of endophytic fungi in the same regions showed high similarity in species taxonomy [27]. On the contrary, even among the same host plants from different regions, the species and population structure of endophytic fungi usually showed very low similarity [26].

Although endophytic fungi are one of the most important elements in plant microecosystems and play an important role in the growth and development of parasitic plants, the exact relationship between them and parasitic plants is still very limited. The population structure and distribution patterns of endophytic fungi is closely related to environmental changes, classification of host plants and genetic background. The results showed that the distribution patterns of endophytic fungi were significantly affected by environmental conditions such as temperature, humidity, light, geographical location and the surrounding vegetation [28–29]. In addition, the growth stage configuration (age) of host plants and tissues may also affect the species composition of endophytic communities [30]. For example, different endophytic species have been found in tissues such as parenchyma, vascular and dermis of different aged host plants of [31]. Therefore, understanding the distribution and population structure patterns of endophytic fungi can provide theoretical guidance for the effective exploration of pharmaceutical bioactive compounds produced by specific host medicinal plants in specific tissues and under specific environmental conditions.

There are relatively few reports on the diversity of endophytic fungi in parasitic plants, and the distribution and community structure of these fungi have not been systematically analyzed. Lu et al. isolated an endophytic fungus, *Alternaria*, from the parasitic leaves of *S. chinensis*, and its metabolites showed some antioxidant and antibacterial activities [32]. Xue isolated eight endophytic fungi from *Cynomorium songaricum* at different developmental stages. These belong to *Aspergillus*, Dothideomycetes, Sordariomycetes, *Fusarium*, *Gibberella*, *Lentinula*, Plectosphaerellaceae and *Candida*, respectively. Correlation analysis between the endophytic fungi and the differential metabolites of *C. songaricum* at different growth stages showed that there was a significant correlation between the fungi and some differential secondary metabolites at different concentrations, indicating that endophytic fungi play an important role in the accumulation, production and other life activities of *C. songaricum* [33]. Bao et al. found that the total number of root and functional haustoria of the endophytic fungus *Epichloë inebrians* that were infected with the host, *Stipa purpureata*, were significantly higher than those seen in a single species [34].

Taxillus chinensis belonging to the Loranthaceae family are mainly distributed in the southern and southwestern areas of China. The dry stems and branches with leaves of *T. chinensis* are commonly used as materials for traditional Chinese medicine, which is referred to as “Sang Ji Sheng” in China. *T. chinensis* has a high medicinal value. It is used for relief from rheumatic conditions, reinforcement of the liver and kidney and strengthening of the tendons and bones as well as prevention of abortion [35]. Meanwhile, *T. chinensis* is also used as raw materials for making parasitism tea in China, and it is exported to nearly 30 countries in Southeast Asia [36]. While there is an increasing market demand, it also has a very important position in Guangxi and it is even sold on the national Chinese herbal medicine markets.

Relatively few studies have been performed on the endophytic fungi of the genus *Pestalotiopsis*. Gong et al. isolated an endophytic fungus of the genus *Pestalotiopsis* from the *T. chinensis*, which had significant inhibitory effects on cultured A549 and H460 tumor cells[37].

Therefore, in this study 7 host species, including *Morus alba*, *Prunus salicina*, *Phellodendron chinense*, *Dalbergia odorifera*, *Bauhinia purpurea*, *Diospyros kaki* and *Dimocarpus longan* were selected as branches of *T. chinensis*, respectively. These were used to separate the endophytic fungi of *T. chinensis*, in order to analyze the diversity of these fungi on different hosts of this species. We also evaluated their antagonistic effects against three important plant pathogens, *Corynespora cassicola* of *Sarcandra glabra*, *Didymella glomerata* of *Sophora tonkinensis* and *Stagonosporopsis cucurbitacearum* of *Siraitia grosvenorii*. This provided valuable information regarding the distribution of the endophytic fungi in *T. chinensis*, and will allow basic research into conservation of this valuable resource.

Results

IR of endophytic fungi

A total of 150 endophytic fungi strains were isolated and purified from 245 tissue segments from *T. chinensis* parasitized on different host plants and the average IR of the endophytic fungi from *T. chinensis* was 61.22%. However, the IR of endophytic fungi from *T. chinensis* varied greatly due to the differences of host plants species. The highest IR of endophytic fungi was obtained from *T. chinensis* parasitized on *M. alba*, and this was 91.43%. However, the lowest IR of endophytic fungi was obtained from *T. chinensis* parasitized on *P. chinense* was only 28.57% (Fig. 2 and Table S1).

Colony composition of endophytic fungi of T. chinensis

In combination with the characteristics of morphology and molecular biology of the isolated strains to determine the status of classification, the results showed that there were 150 strains of endophytic fungi as well as three strains which were not clearly identified. The overall IR was 61.24% (Table 1). Other strains belonged to 1 phylum, 2 classes, 7 orders, 9 families, 11 genera and 12 species (Fig. 3), including ascomycetes, Ascomycota. Sordariomycetes was the dominant group, accounting for 96.00% of the total number of strains. At the orders level, the dominant order was Xylariales, accounting for 46.67% of the total number of strains (Fig. 4-A and Table s2). At the genus level, *Pestalotiopsis*, *Neopestalotiopsis* and *Diaporthe* were the dominant genera, accounting for 26.67, 17.33 and 31.33% of the total strains, respectively (Fig. 4-B). *Nigrospora*, *Xylaria*, *Fusarium* and *Exserohilum* were the common genera, accounting for 7.33, 1.33, 1.33, 1.33% of the total strains, respectively (Fig. 4-B). *Hypoxylon*, *Daldinia*, *Colletotrichum* and *Neofusicoccum* were rare genera, accounting for 0.67% of the total strains (Table 1, Fig. 4-B and Table s3). Sequences of these twelve strains were submitted to the GenBank database, and the accession numbers obtained were MZ836840, MZ823600, MZ2823598, MZ836841, MZ836842, MZ836843, MZ823601, MZ836844, MZ823599, MZ836845, MZ823597 and MZ836846 for strains 1, 15, P6, 20, 17, 24, 4, 13, N6, 22, 31 and 9, respectively.

Table 1
Community composition of the endophytic fungi of different hosts of Taxillus chinensis.

Phylum	Class	Order	Family	Genus	Species	N	IF (%)
Ascomycota	Sordariomycetes	Trichosphaeriales	Trichosphaeriaceae	*Nigrospora*	*Nigrospora* spp.	10	6.67
					Nigrospora sphaerica	1	0.67
		Xylariales	Sporocadaceae	*Pestalotiopsis*	*Pestalotiopsis* spp.	40	26.66
				Neopestalotiopsis	*Neopestalotiopsis* spp.	26	17.33
			Xylariaceae	*Xylaria*	*Xylaria longipes*	1	0.67
					Xylaria sp.	1	0.67
			Hypoxylaceae	*Hypoxylon*	*Hypoxylon investiens*	1	0.67
				Daldinia	*Daldinia govorovae*	1	0.67
		Glomerellales	Glomerellaceae	*Colletotrichum*	*Colletotrichum* sp.	1	0.67
		Hypocreales	Nectriaceae	*Fusarium*	*Fusarium incarnatum*	1	0.67
					Fusarium sp.	1	0.67
		Diaporthales	Diaporthaceae	*Diaporthe*	*Diaporthe* spp.	58	38.66
					Diaporthe perseae	2	1.33
	Dothideomycetes	Pleosporales	Pleosporaceae	*Exserohilum*	*Exserohilum rostratum*	2	1.33
		Botryosphaeriales	Botryosphaeriaceae	*Neofusicoccum*	*Neofusicoccum parvum*	1	0.67
	Unclassidied fungi			Unidentified		3	2.00
Total						150	100
Note: N is the number of strains; IF is the isolation frequency							

IR of endophytic fungi in different hosts of T. chinensis

The number of endophytic fungal strains isolated from *T. chinensis* branches of different hosts was different, and the number of endophytic fungal strains isolated from *M. alba* was the highest with up to 32. This was followed by *D. odorifera*, *D. longan*, *B. purpurea*, *P. salicina* and *D. Kaki* which consisted of 27, 26, 23, 20 and 12 strains, respectively. Only 10 were isolated from *P. chinense*.

The IFs of endophytic fungi from the branches of *T. chinensis* of the seven hosts were also analyzed. The most frequently isolated endophytic fungi were *Pestalotiopsis* and *Neopestalotiopsis* in the parasitic branches of *T. chinensis* from *M. Alba*, *D. odorifera*, *D. Longan*, *P. salicina* and *D. Kaki*. Among them, *M. alba* and *D. odorifera* had the highest separation frequencies with both reaching 7.33%. In addition to *P. chinense*, the endophytic fungi with the highest IF in the parasitic branches of the other 6 hosts were *Diaporthe*, among which *D. odorifera* had the highest IF of up to 12%, followed by *B. purpurea* which was up to 10% (Fig. 5 and Table s4).

Diversity of endophytic fungi of T. chinensis **in different hosts**

Table 2 shows the diversity index of endophytic fungi of the haustoria of *T. chinense* that parasitized the seven plants, *D. longan*(1.60) > *D. odorifera* 1.51) > *M. alba* (1.41) > *P. chinense* (1.16) > *B. purpurea* (1.03) > *P. salicina* > (0.86) > *D. kaki* (0.77. The richness indexes were calculated to be *M. alba* (2.23) = *P. chinense* (2.23) > *D. longan* (1.97) > *D. odorifera* (1.95) > *B. purpurea* > (1.29) > *D. kaki* (1.11) > *P. salicina* (0.69). The evenness indexes were *D. longan* (0.82) > *P. salicina* (0.79) > *D. odorifera* (0.73) > *P. chinense* (0.72) > *M. alba* (0.68) > *B. purpurea* (0.64) > *D. kaki* (0.56). The results showed that *D. longan* had the largest diversity index (1.60), with the largest richness index (2.23) of endophytic fungi of the haustoria found in *M. alba* and *P. chinense*. *D. longan* also had the largest evenness index (0.82).

Table 2
The diversity indexes of endophytic fungi of different hosts of T. chinensis.

Host	Shannon-Weiner	Margalef's	Evenness
	diversity index (H')	index (D)	index
M. alba	1.41	2.23	0.68
P. salicina	0.86	0.69	0.79
P. chinense	1.16	2.23	0.72
D. odorifera	1.51	1.95	0.73
B. purpurea	1.03	1.29	0.64
D. kaki	0.77	1.11	0.56
D. longan	1.60	1.97	0.82

Similarity coefficients of endophytic fungi of T. chinensis **in different hosts**

Table 3 shows that the similarity coefficients of endophytic fungi of the haustoria of *T. chinense* that parasitized different hosts were different. The similarity coefficient between *D. longan* and *D. kaki* was the highest, which was 36.36%. The similarity coefficients of *D. longan*, *M. alba* and *D. odorifera* were 33.33% and those of *P. salicina* and *D. kaki* was 28.57%. The similarity coefficient of *D. longan* and *M. alba* was 26.67%. The similarity coefficient was the lowest with *M. alba* and *P. chinense* with *D. odorifera* (7.69%), while there was no similarity coefficient with *P. salicina* and *D. kaki*, indicating that there were no similar endophytic fungi of the haustoria of *T. chinense* between *P. salicina* and *D. kaki* with *P. salicina*.

Table 3
The similarity coefficients of endophytic fungi of different hosts of T. chinensis (%).

Hosts	*M. alba*	*P. salicina*	*P. chinense*	*D. odorifera*	*B. purpurea*	*D. kaki*	*D. longan*
M. alba							
P. salicina	18.18						
P. chinense	7.69	0					
D. odorifera	33.33	18.18	7.69				
B. purpurea	15.38	12.5	10	23.08			
D. kaki	25	28.57	0	25	22.22		
D. longan	26.67	20	17	33.33	25	36.36	

In vitro **antagonistic assays of endophytic fungi against phytopathogens**

The antagonistic activity of 9 fungal endophytes against the plant pathogens *C. cassicola*, *D. glomerata*, *S. cucurbitacearum* was evaluated in co-culture tests (Table 4). These 9 strains of endophytic fungi had different degrees of inhibition on these three pathogens, and the inhibition rates were above 60%. Among the 11 strains, *Neofusicoccum parvum* showed a significant antagonistic activity against *D. glomerata* and *C. cassicola*, and the inhibition rates were 85.29% and 78.82%, respectively. However, there was no inhibition on this with *S. cucurbitacearum*. The species, *Hypoxylon investiens*, was the one that had the best inhibition of *S. cucurbitacearum*. The inhibition rate was 83.53%.

Table 4
Inhibition of endophytic fungi on different pathogens found in medicinal plants.

Strain	Species	Mean percentage of mycelial growth inhibition (%)		
		D. glomerata	*C. cassicola*	*S. cucurbitacearum*
4	*Colletotrichum* sp.	$(68.82 \pm 1.70)^{a}$	$(60.00 \pm 1.18)^{a}$	$(69.41 \pm 2.35)^{a}$
15	*Nigrospora sphaerica*	$(70.59 \pm 0.68)^{ab}$	$(62.35 \pm 1.18)^{ab}$	$(62.35 \pm 1.18)^{b}$
1	*Nigrospora* sp.	$(71.76 \pm 1.36)^{ac}$	$(75.88 \pm 1.76)^{c}$	$(67.65 \pm 0.59)^{ac}$
N6	*Diaporthe phaseolorum*	$(73.53 \pm 1.70)^{bc}$	$(70.00 \pm 4.12)^{d}$	$(74.12 \pm 1.18)^{d}$
P6	*Pestalotiopsis* sp.	$(75.29 \pm 2.03)^{cd}$	$(71.18 \pm 0.59)^{de}$	$(75.88 \pm 0.59)^{de}$
9	*Neofusicoccum parvum*	$(85.29 \pm 1.02)^{e}$	$(78.82 \pm 2.35)^{cf}$	-
17	*Hypoxylon investiens*	$(72.35 \pm 0.34)^{abcd}$	$(69.41 \pm 1.18)^{deg}$	$(83.53 \pm 2.35)^{f}$
31	*Erostratum rostratum*	$(74.71 \pm 1.02)^{cd}$	$(72.94 \pm 1.18)^{cdegh}$	$(72.35 \pm 2.94)^{adg}$
22	*Diaporthe perseae*	$(78.82 \pm 0.00)^{df}$	$(62.94 \pm 4.12)^{abi}$	$(75.29 \pm 1.18)^{degh}$
Different lowercase letters in the same column represent a significant difference ($P < 0.05$).				

Antimicrobial activity of crude extracts from fermentation products of endophytic fungi

As can be seen from Fig. 6, the crude metabolite extracts of the endophytic fungi strains 9, 17 and P6 of *T. Chinensis* seeds had obvious inhibitory effects on these three pathogens, and they can be used as fermentation production strains. The antibacterial activities of the crude extracts of fermentation products of strains 9, 17 and P6 are shown in Fig. 6. As shown in Fig. 6, when the concentration of crude extracts was 100 mg/mL and they were cultured for 11 days, the results showed that they could significantly inhibit the growth of *D. glomerata*, *C. cassicola* and *S. cucurbitacearum*. Among these, strains 9, 17 and P6 had the strongest inhibitory effects of *S. cucurbitacearum* in Table 5, with inhibitory rates of 100%, 100% and 81.51%, respectively. Secondly, strain 9 had a strong inhibitory effect on *D. glomerata* and *C. cassicola*, with inhibitory rates of 82.35% and 72.80%, respectively. The extracts of strains 9, 17 and P6 were 6.456 g, 11.069 g and 9.727g, respectively.

Table 5
The antibacterial activities of crude extracts from fermentation products of endophytic fungi.

Strain	Species	Mean percentage of mycelial growth inhibition (%)		
		D. glomerata	*C. cassicola*	*S. cucurbitacearum*
9	*Neofusicoccum parvum*	$(82.35 \pm 1.22)^{a}$	$(72.80 \pm 3.21)^{acd}$	$(100 \pm 0)^{g}$
17	*Hypoxylon investiens*	$(66.91 \pm 0.85)^{b}$	$(66.47 \pm 0.34)^{be}$	$(100 \pm 0)^{gh}$
P6	*Pestalotiopsis* sp.	$(77.45 \pm 2.16)^{ac}$	$(70.35 \pm 3.29)^{abcdf}$	$(81.51 \pm 0.42)^{aci}$

Discussion

In this study, a total of 150 endophytic fungi were isolated from healthy haustorial roots of *T. chinensis* of 7 hosts from the same habitat in Guangxi, and they belonged to 11 genera. Among them, *Pestalotiopsis*, *Neopestalotiopsis* and *Diaporthe* had high IFs and were widely distributed in these hosts. As the dominant genus of endophytic fungi, these results are similar to those reported for endophytic fungi in *Taxilli herba* from *Salix babylonica* in Guangxi [37]. There were some differences in the distribution of endophytic fungi in the haustoria of *T. chinensis* of different hosts. Four species, *Exserohilum rostratum*, *Neofusicoccum parvum*, *Hypoxylon investiens* and *Nigrospora musae*, have only been isolated from the haustorial roots of *P. chinense*. *Daldinia govorovae*, was isolated only from the haustorial roots of *M. longan*. The two species, *Xylaria longipes and Diaporthe perseae*, were isolated only from the haustorial roots of *B. purpurea*. *Nigrospora sphaerica, Xylaria* sp. and *Fusarium* sp.. The two species, *Colletotrichum acutatum and Fusarium incarnatum*, were isolated only from the haustorial roots of *D. odorifera*. All these results indicated that the distribution of endophytic fungi had some host specificity [38–40]. When

combined with the results of diversity and similarity coefficient analysis of endophytic fungi of the seven hosts, it was found that there were some differences among the endophytic fungi of the haustoria of *T. chinensis* in different plants. Since samples were collected from the same places and the same habitats in this study, the results strongly indicated that there might be some preferences for endophytic fungi of haustorial roots of *T. chinensis* different hosts.

In this study, samples were collected from parasitical branches of seven hosts in the same habitat and the same season (winter), and the parasitical branches of *D. longan* were more abundant in endophytic fungi. The species and quantity of endophytic fungi in medicinal plants were rich and varied in the different hosts, and the endophytic fungi in medicinal plants are also likely to change with the changes of regions, seasons, growth stage, tissues and organs [41–42]. Lü et al. (2014) showed that the IR of endophytic fungi in *Atractylodes lancea* of Maoshan type was higher than that in *A. lancea* of Hu Bei type, and the composition of fungal diversity was also partially different [43]. With the change of seasons, the endophytic fungal community showed certain succession rules, and the diversity of endophytic fungi in summer was higher than that in spring and autumn. Shu et al. found that the number and species of endophytic fungi in branches were more than those in leaves and fruits in different tissues of *Citrus maxima* [44]. Wang et al. showed that the abundance and diversity of fungi in the wild environment were higher than those in a park environment for *Paris polyphyllain* [41–42]. Ren et al. found that with the increase of altitude, the diversity of endophytic fungi in the roots of *Rhododendron simsii* was less varied, the community distribution was more uneven and the dominant fungi were more prominent [45]. In addition, the species of dominant endophytic fungi were geographically different [46–47]. Xu et al. found that the mycorrhizal fungal community diversity of *Cypripedium tibeticum* (Huanglong Gully, Sichuan) at different altitudes also decreased with an increase of altitude [47].

Endophytic fungi of medicinal plants can inhibit fungi and prevent diseases, have a broad spectrum of inhibition and have the potential to develop biological control substances [48–49]. In this work, *N. parvum* and *H. investiens* showed significant antifungal activity against three fungal phytopathogens of medicinal plants. *N. parvum* and *H. investiens* are tall and they are able to occupy space and absorb nutrients rapidly, so as to effectively compete with pathogens in nutrition and space and inhibit their growth. Therefore, future investigations will be conducted to study their potential as biocontrol agents on an agronomic scale.

The next step of this research is to explore the gathering of specimens from different habitats and different altitudes, different organizations and different seasons with regard to the endophytic fungi diversity and similarity coefficient of *T. chinensis*, in order to further understand the distribution of these fungi in *T. chinensis*. This type of knowledge can allow the mining of more endophytic fungi resources, thus illustrating the ecological function and dynamic distribution of endophytic fungi in *T. chinensis*.

Conclusions

The diversity of endophytic fungi from different hosts of *T. chinensis* showed the diversity values which varied significantly, and they are the promising antagonists by competition with plant pathogens. These microorganisms can be a source for new metabolites for use in biocontrol and these fungi. They can also provide highly efficient strains that will be useful in industrial processes in the future.

Materials And Methods

The sample collection of T. chinensis

In January 2020, the haustoria of *T. chinensis* from different hosts including *M. alba*, *P. salicina*, *P. chinense*, *D. odorifera*, *B. purpurea*, *D. kaki* and *D. longann* were collected in the *T. chinensis* planting base of Cenxi Funing Village, Wuzhou, Wuzhou City (111°51'14"E, 22°58'12"N) (Fig. 1). The seven different hosts were situated within 20 meters of each other. We collected 3–5 haustoria of *T. chinensis* from the same host plants and were placed in labelled, sealed bags and then they were returned to the laboratory for endophytic fungi isolation within 48 hours of collection.

Isolation And Purification Of Endophytic Fungi

Healthy and disease-free haustorial roots of *T. chinensis* from different hosts were selected and the tissues were cut into 5cm fragments [50]. The surfaces were successively disinfected with 75% ethanol and 0.1% Hg for 2.5min, and were washed with sterile water 3 times. Using sterile forceps and scalpels, they were cut into tissue blocks of about 5 mm in size, and then placed on PDA medium containing streptomycin and plated with 7 blocks per plate and each sample consisted of 5 plates. In order to ensure the accurate separation of endophytic fungi of *T. chinensis*, asepsis detection methods on extra-clean workbenches were used throughout [51]. The cultures were conducted at 28℃ until the mycelia grew from the edges of the tissue blocks, and these were then transferred to PDA plates for culture, purification and preservation. The

isolated and purified endophytic fungi were divided into different morphological types according to the characteristics of the culture colonies [9].

Identification Of Endophytic Fungi

The colony morphology was recorded by referring to Fang's method [50], and the preliminary identification of colony morphology was conducted by using Wei's method [52] as well as the use of the international classification website (http://www.indexfungorum.org). For molecular biological identification, a MightyAmp DNA Polymerase Ver.3 (1.25 U/50 μL) kit (Takara Bio Inc., Japan, Cat. No. R076A) was used. Universal primers ITS1 and ITS4 [53] were used for sequence amplification. Colonies of cultured endophytic fungal hyphae were selected as templates for direct PCR reactions. The target bands were detected by gel imaging methods, and the PCR products of the target bands were sent to BGI (Guangzhou) Co. Ltd. for sequencing. The sequencing results were compared with the sequences in the GenBank of NCBI using BLAST. The linking method of MEGA 7.0 software was used to construct an ITS rDNA phylogenetic tree by employing the method of Kumar [54]. This allowed the similarity between a target sequence and a phylogenetically relevant referenced 95% sequence to be identified and these were then classified as an unidentified strain. More than 95% of the strains were identified to the genus level and more than 97% were identified to the species level. In addition, it was found that more than 99% were identified as the same species [55].

Diversity Analysis And Data Processing

Isolation Rate (IR): This refers to the percentage of the number of isolated strains in the sample tissue blocks to the total number of sample tissue blocks, and this was used to measure the abundance of endophytic fungi in plant tissues and the occurrence frequency of multiple infections in each tissue block.

Isolation frequency (IF): This refers to the percentage of the number of isolated endophytic fungi strains to the total number of isolated endophytic fungi, and this was used to reflect the dominance of endophytic fungi of different species (classes) in the total flora. IF ≥ 10% was the dominant genus, IF ≥ 1% was the common genus, IF < 1% are rare genera [56].

Shannon-Wiener (H') = -Σ(PilnPi), where Pi refers to the percentage of the number of a certain strain of endophytic fungi to the total number of all isolated endophytic fungi, and this was used to analyze the diversity of endophytic fungi and the complexity of the community in a specific community [57].

Species richness index (Margalef's index, D): D= (total number of taxa − 1)/ln (total number of individuals), was used to analyze the richness of taxa [58].

Evenness index (E): E = H'/Ln(S), where H' is Shannon-Wiener index and S was the number of species found, and this was used to analyze the evenness of the distribution of endophytic fungi of different species in different hosts.

Sorenson's similarity coefficients (Cs) were used to evaluate the similarity of the species composition of endophytic fungi between two hosts. The formula used to obtained this was Cs = 2j/(a + b), where j is the number of species shared by the two populations, a is the number of all the species in the first population and b is the number of all the species in the second population.

In vitro **antagonistic assays of endophytes against fungal phytopathogens**

The *in vitro* antagonistic activity of endophytic fungi against *Corynespora cassicola* of *Sarcandra glabra*, *Didymella glomerata* of *Sophora tonkinensis* and *Stagonosporopsis cucurbitacearum* of *Siraitia grosvenorii* were tested using the co-culture method [59–60]. Briefly, one mycelial plug (6 mm diam) of each 7-d-old fungal phytopathogen was placed at the center of a dish containing approximately 9 mL of PDA, yielding a final depth of 2 mm. Two mycelial plugs (6 mm diam) from one 7-d-old endophytic fungus were symmetrically placed 2 cm from the endophytic inoculant to establish a co-culture treatment. Plates containing only pathogens were used as the controls. All treatments and controls were run in duplicate and incubated at 28 ℃. When the fungal phytopathogen colony had completely reached the center of the petri dishes in the growth control, the radius of the relative fungal phytopathogen colony in the treatment dishes was measured. The average radius of each fungal phytopathogen in the treatment was recorded as R1, and that in the growth control was recorded as R2. The growth inhibition percentage of the fungal phytopathogen with respect to the endophyte – phytopathogen antagonism was calculated with the formula as mentioned below:

$$\text{Inhibition percentage}(\%) = \frac{\text{R}_2\text{-R}_1}{\text{R}_2} \times 100$$

Crude Extract Preparation Of The Fungal Fermentation Broth

The seed endophytic fungus with the best antibacterial activity was selected as the production strain for small-scale solid fermentation. The production strains were seeded on PDA culture plates and incubated at 28°C for 5 days. After colony maturation, 5 truffle cakes with a diameter of 6 mm were inoculated into 50 mL triangular vials containing 20 mL of liquid potato medium as seed strains for culture. A total of 2 vials were inoculated and incubated at 28°C for 3d by shaking at 120 rpm. 60g of rice was added to a 500mL triangular bottle, 90mL was steamed with water, autoclaved for 20 min, cooled and placed under sterile conditions. 10 mL of seed bacteria liquid was poured into 500 mL triangular bottles containing solid medium with a total of 4 bottles. These were mixed well and covered with 8 layers of gauze, and incubate at 28°C. After 30 days, the gauze was removed and methanol poured in. The mixture was soaked and fermented and the liquid level was seen to increase by 1-2cm. This was further soaked for 40 min and then subjected to ultrasonic extraction for 20 min. The mixture was filtered and this procedure was repeated for 3 times. The combined filtrate was kept at 45°C constant temperature in a water bath and then this was subjected to rotary evaporation to dry the extract [61].

Antibacterial Activity Of Crude Extracts Of Fermentation Products

The crude extract of 400 mg fermentation product was weighed into a 5 mL sterilized centrifuge tube, and 4 mL of methanol was added to completely dissolve it in order to prepare a crude extract solution of 100 mg/mL. This was sterilized by filtration using a Millipore filter (0.22μm) prior to antimicrobial assays. 1 mL of each solution was absorbed and coated with a pipette gun, and three plates were repeated for each gradient. Under sterile conditions, the methanol in the plate was evaporated, and the pathogenic fungi were inoculated in the center of the PDA plate. The cultures were incubated at 28°C for 5 days, and the growth of the three pathogenic fungi were observed.

Statistical Analysis

Statistical results were expressed as $x \pm s$, with ± being the mean value and s being the standard deviation. SPSS19.0 software was used to conduct univariate ANOVA analysis and the variance homogeneity test for data in each group. $P < 0.05$ was considered statistically significant.

Abbreviations

PCR
Polymerase chain reaction
PDA
Potato Dextrose Agar
PDB
Potato Dextrose Broth
IF
Isolation frequency
IR
Isolation Rate.

Declarations

Ethics approval and consent to participate

The endophytic fungal strains isolated from seven hosts (*M. alba*, *P. salicina*, *P. chinense*, *B. purpurea*, *D. odorifera*, *D. kaki* and *D. longan*) were kept in the plant Pathology Department of Guangxi Botanical Garden of Medicinal Plants and we have obtained written consent from plant Pathology Department of Guangxi Botanical Garden of Medicinal Plants to use these fungal strains in our research. The experimental methods conducted in this study complied with current Chinese laws and regulations.

Consent for publication

Not applicable.

Availability of Data and Material (ADM)

Sequences of these twelve strains were submitted to the GenBank database, and the accession numbers obtained were MZ836840, MZ823600, MZ2823598, MZ836841, MZ836842, MZ836843, MZ823601, MZ836844, MZ823599, MZ836845, MZ823597 and MZ836846 for strains 1, 15, P6, 20, 17, 24, 4, 13, N6, 22, 31 and 9, respectively. Other data generated or analyzed during this study are included in the article and its supplementary information files.

https://www.ncbi.nlm.nih.gov/search/all/?term=MZ836840;https://www.ncbi.nlm.nih.gov/search/all/?term=MZ823600;https://www.ncbi.nlm.nih.gov/pmc/?term=MZ2823598;https://www.ncbi.nlm.nih.gov/search/all/?term=MZ836841;https://www.ncbi.nlm.nih.gov/search/all/?term=MZ836842;https://www.ncbi.nlm.nih.gov/search/all/?term=MZ836843;https://www.ncbi.nlm.nih.gov/search/all/?term=MZ823601;https://www.ncbi.nlm.nih.gov/search/all/?term=MZ836844;https://www.ncbi.nlm.nih.gov/search/all/?term=MZ823599;https://www.ncbi.nlm.nih.gov/search/all/?term=MZ836845;https://www.ncbi.nlm.nih.gov/search/all/?term=MZ823597;https://www.ncbi.nlm.nih.gov/search/all/?term=MZ836846

Conflict of interest

The authors declare no competing interests.

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Author contributions

SGW and JEF: Reviewed and finalized manuscript, LSS: completed the Article writing, NJ, LLH and LMP: integrated information of the tables, JH: analysis of data and construction of the Figures, LYW: completed writing of the manuscript. The final manuscript was approved by all authors who agreed to be accountable for the content of this work.

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Supplementary information

Additional files 1: Table S1. figure 2 raw data; Table S2-S3. figure 4A and 4B raw data; Table S4. figure 5 raw data.

Additional files 2: Figure 3 raw data. Phylogenetic tree powerpoint.

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Figures

Figure 1

Maps showing the locations of the sample collection sites

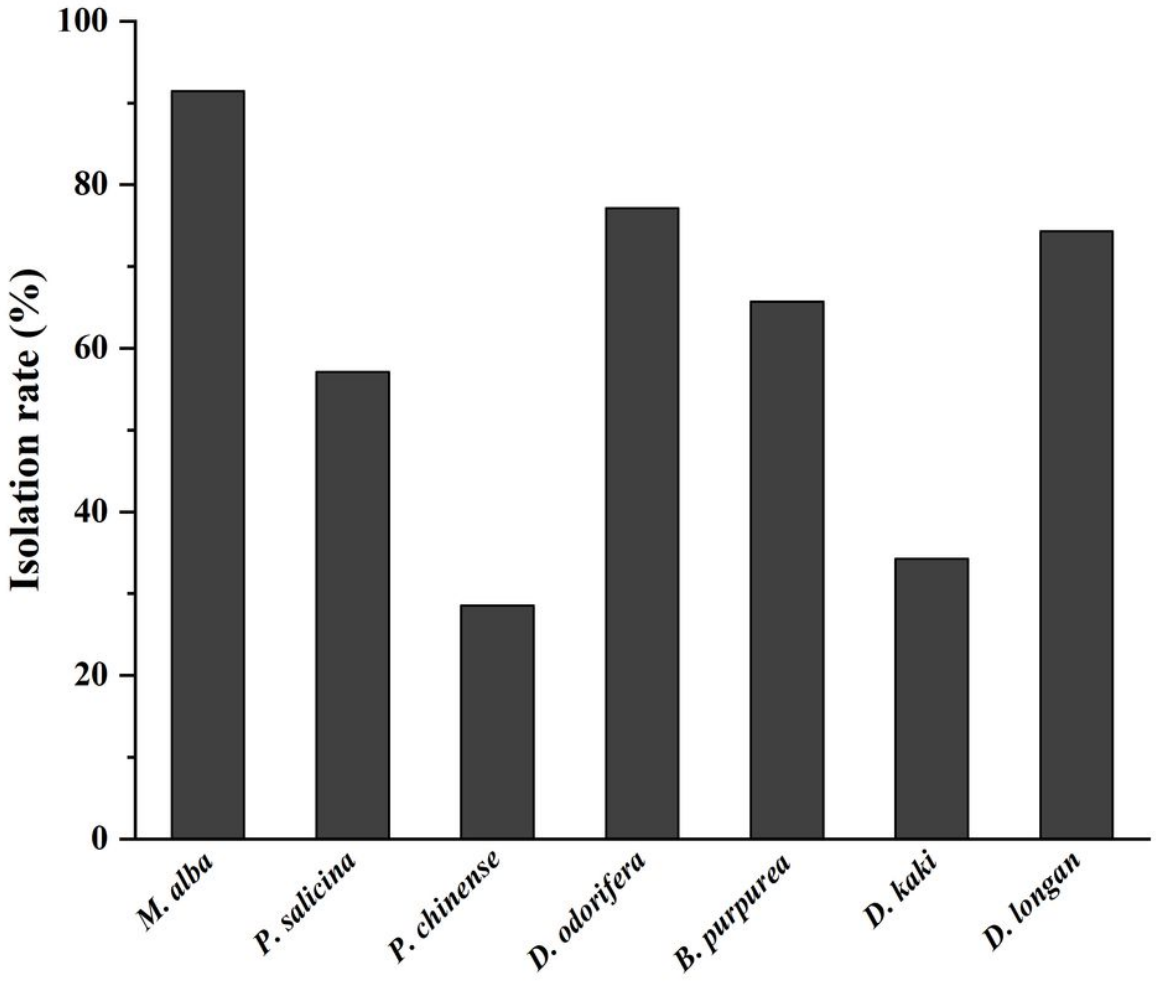

Figure 2

The isolation rates of endophytic fungi from *T. chinensis*

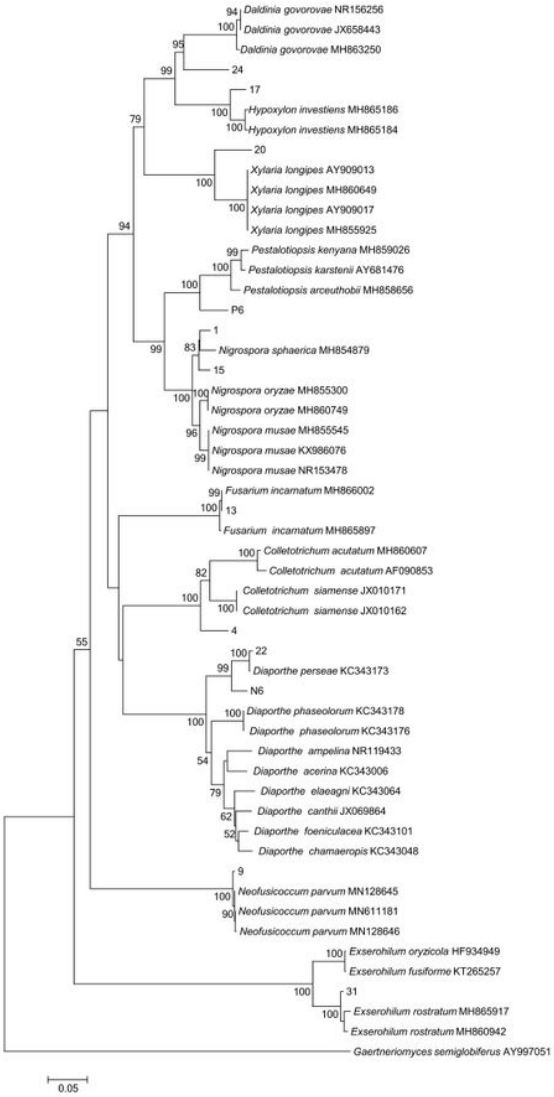

Figure 3

Phylogenetic analysis of endophytic fungi from *T. chinensis* parasitized on different host plants based on rDNA-ITS

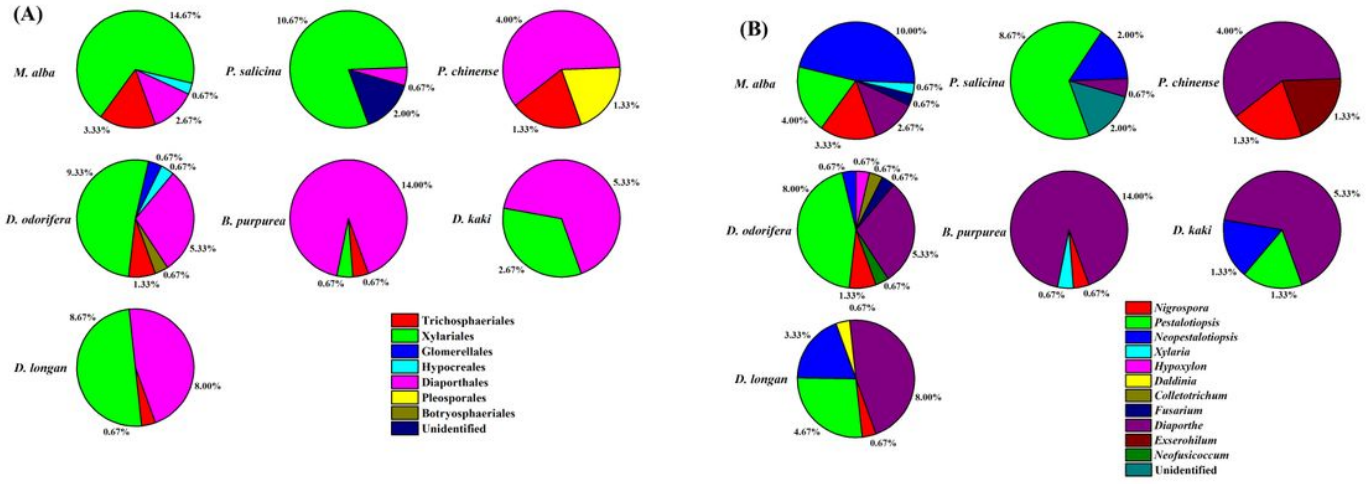

Figure 4

The isolation frequency of orders (A) and genera (B) related to *Taxillus chinensis* parasitized on different host plants

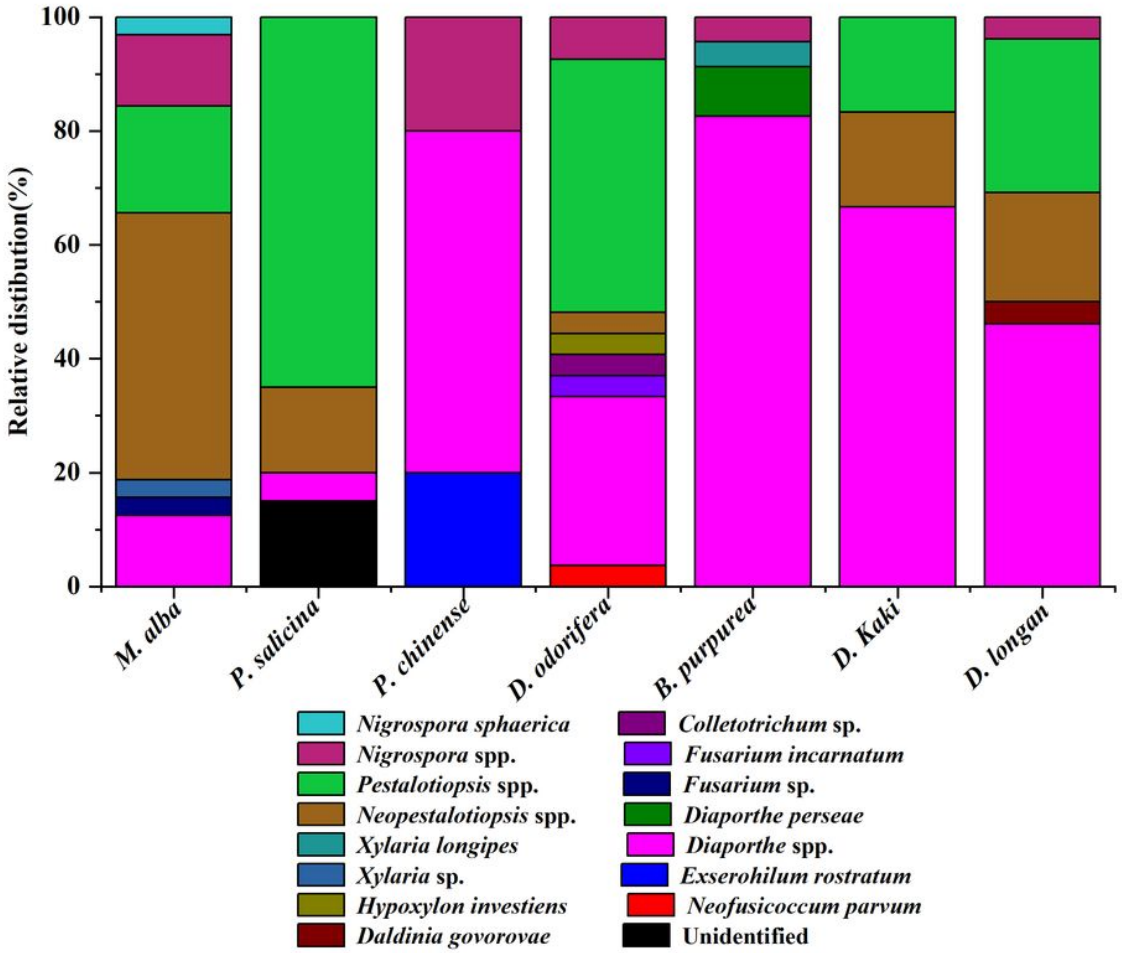

Figure 5

The diversity and distribution of endophytic fungi isolated from *Taxillus chinensis* parasitized on different host plants

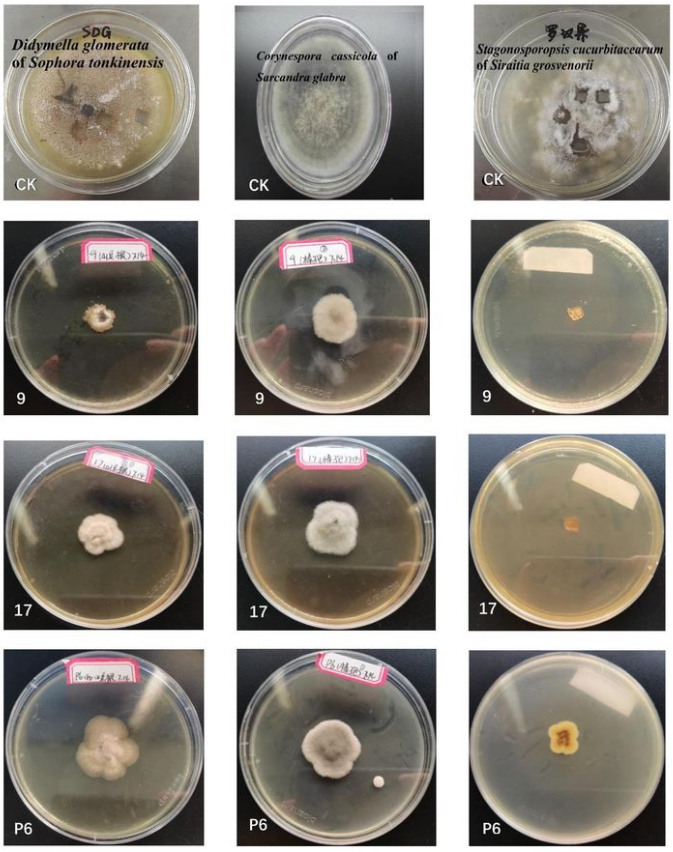

Figure 6

The inhibitory effects of crude extracts from fermentation products of endophytic fungi

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- tables1figure2rawdata.xlsx
- tables2Figure4Arawdata.xlsx
- tables3Figure4Brawdata.xlsx
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