Diversity and bioactivities of fungal endophytes from Distylium chinense, a rare waterlogging tolerant plant endemic to the Three Gorges Reservoir

Xiaoxiang Duan
Chongqing Academy of Chinese Materia Medica
Fangfang Xu
Southwest University
Dan Qin
Southwest University
Tiancong Gao
Southwest University
Weiyun Shen
Huzhou University
Shihao Zuo
Southwest University
Baohong Yu
Southwest University
Jieru Xu
Southwest University
Yajun Peng
Southwest University
Jinyan Dong (donjyaa@swu.edu.cn)
Southwest University

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Abstract

Background The present study focuses on diversity and biological activities of the endophytic fungal community from Distylium chinense , a rare waterlogging tolerant plant endemic to the Three Gorges Reservoir. This study has been explored the characteristics of endophytic fungi in waterlogged environment including both aquatic and terrestrial fungi, and they may produce new metabolites under complex and extreme conditions, which may possess bioactive property. Therefore, the antioxidant, antimicrobial and anticancer activities of all endophytes isolated from this study have been investigated. Moreover, the active metabolites of the most broad-spectrum bioactive strain have also been studied.

Results A total of 154 fungal endophytes were isolated from roots and stems. They were categorized into 30 morphotypes based on cultural characteristics and were affiliated with 27 different taxa. Among these, the most abundant fungal orders included Diaporthales (34.4%) and Botryosphaeriales (30.5%), which were predominantly represented by the species Phomopsis sp. (24.7%) and Neofusicoccum parvum (23.4%). Fermentation extracts were evaluated, screening for antioxidant, antimicrobial and anticancer activities. Among the 154 isolates tested, 99 (64.3%) displayed significant antioxidant activity, 153 (99.4%) exhibited inclusive antimicrobial activity against at least one tested microorganism and 27 (17.5%) showed exclusive anticancer activity against one or more cancer cell lines. Specifically, the crude extract of Irpex lacteus DR10-1 exhibited note-worthy bioactivities. Further chemical investigation on DR10-1 strain resulted in the isolation and identification of two known bioactive metabolites, indole-3-carboxylic acid (1) and indole-3-carboxaldehyde (2), indicating their potential roles in plant growth promotion and human medicinal value.

Conclusions These results indicated that diverse endophytic fungal population inhabits D. chinense . The isolated endophyte DR10-1 (Irpex lacteus) has the potential to be a source of novel antioxidant/antimicrobial/anticancer compounds. The findings of the present study not only provide a sustainable resource for the utilization of endophytic fungi in D. chinense but also provides an important basis for further understanding of fungal communities in medicinal plants.

Background

Endophytic fungi in plants are microorganisms that parasitize symbiotically in the internal tissues during the whole or part of their life cycles of the hosts without causing apparent pathogenic symptoms [1], but may turn pathogenic during host senescence [2]. Accumulated evidence has confirmed that plant endophytes from special orextreme environment has many effects on host ecological adaptability [3–5]. It is well known that the concurrence of endophytes may accelerate plant growth and increase the survival rate of biotic or abioticstresses, such as plant diseases, pests, drought, salinity and extreme temperatures [6–9]. Specifically, some endophytes are beneficial to plants by producing special substances, such as secondary metabolites, which can prevent the host from being attacked successfully by fungi and pests [10]. So far, endophytes, especially those under complex and extreme conditions, have been shown to produce a variety of metabolites with complex structures, such as alkaloids, terpenoids,

polyketides, lipids, proteins, glycosides, isoprenoids, and hybrids of those metabolites, etc. [11–13]. More interestingly, these metabolites also showed a variety of interesting bioactivities including antifungal [14], antibacterial [15], anticancer [16], anti-HIV [17], antioxidants [18], etc. Due to these, endophytes from an untapped diverse habitat are a significant source of novel and natural drugs [19].

After Three Gorges Dam was constructed, the Three Gorges Reservoir (TGR) forms a new vast hydro-fluctuation belt with an elevation of 145 m in summer to 175 m in winter, a length of more than 2000 km and an area of 300 km^2 [20, 21], it has formed unique ecological conditions for species diversity and biological distribution in the TGR area [22]. Many field surveys have shown that most of the pre-dam riparian vegetation is gradually dying out due to the inability to adapt to the reversal of submergence time, the prolongation of flood duration and the new hydrological fluctuation zone (up to 30m in elevation) [23]. Generally, plants use limited oxygen and light under flood conditions, resulting in production of excessive reactive oxygen species (ROS) [24], which were the key factors that hindered the growth and development of submerged plants [25, 26]. They are forced to undergo the oxidative pathway [27], and usually develop an antioxidant defense system consisting of some antioxidant enzymes and specific metabolites to convert these excessive ROS into harmless products in order to protect themselves [28, 29].

As symbionts, endophytic fungi can produce antioxidants, block the chain reaction of ROS to help host plants respond to various biotic and abiotic stresses [9, 30]. Some studies have also showed that endophytes can increase the survival rate of host plants during flooding stress by producing antioxidants independently [31, 32]. Severe oxidative damage of free radicals has been confirmed to be associated with various diseases, including cancer, inflammation, aging and neurodegenerative diseases [33]. Thus, antioxidants should be warranted in the enhancement of human health [34, 35]. Currently, the demand for natural antioxidants from endophytic fungi has been increasing along with the finding that natural antioxidants have fewer side effects on human health than artificially synthesized substances [36, 37]. Additionally, the search for safer and novel drugs based on the natural product from endophytes is of utmost importance because of the increasing incidence of cancer and the recently emerged, rapid evolution of superbugs due to antibiotic resistance [38, 39].

It has been reported that only a few highly tolerant plants such as *Salix variegate, Morus alba L., Myricaria laxiflora* etc. survive flooding [22, 40]. Among them, *Distylium chinense* (Fr.) Diels, a rare evergreen perennial shrub of Hamamelidaceae family and endemic to TGR [41] (Fig. 1A), is a native species to the riparian areas and wetlands in the TGR area of the Yangtze River and its tributaries [20]. Additionally, it is an attractive ornamental tree and known for its beautiful and interesting flowers [42]. Its roots are used in traditional Chinese medicine and folk medicine as an analgesic, antirheumatic and diuretic [43]. After completion of the Three Gorges Dam, *D. chinense* was considered as an ideal choice for solid embankment owing to its strong root system, erosion tolerance, strong flooding tolerance and resistance to sand burial soaks [44]. So far, several biological studies have been made for *D. chinense* such as morphological characteristics, natural habitat, genetic diversity, community structure, ecological adaptability, reproductive allocation and propagation methods [42, 45, 46]. However, there is no

information on the diversity and bioactive potential of endophytes community from *D. chinense.* The aim of this study was to provide the first evidence of endophytic fungi diversity within the *D. chinense* to provide a working collection of endophytes and investigate endophytes with antioxidant, antimicrobial and anticancer activities in order to explore the potential sources of novel drugs.

Materials And Methods

Plant material

Three healthy and asymptomatic *D. chinense* plants were randomly collected from different locations on an island in the Banan district (N29º42'45.63", E106º60'69.43") of Chongqing of China in the Three Gorges Reservoir area in October 2014. All plant materials were immediately sent to the laboratory and stored in a refrigerator at 4℃. Each sample tissues were used within 24 h after collection. The plant samples were identified as *D. chinense* by Prof. Hongping Deng and were preserved in Chongqing Key Laboratory of Plant Resource Conservation and Germplasm Innovation, School of Life Science, Southwest University, Chongqing 400715, China.

Isolation and cultivation of endophytic fungi

The surface sterilization and isolation of fungal endophytes were carried out, and some improvements were made [47]. In the first instance, all stems and roots of plant materials were thoroughly washed in running tap water to remove debris and then air-dried naturally in the clean bench. Clean tissue pieces were disinfected in series of solutions: 75% ethanol; sterile distilled water; 0.1% mercuric chloride (HgCl) (v/v). Finally, they were again rinsed with sterile distilled water three times. After surface sterilization, the tissues were dried on blotting sheets, cut into 0.5 cm lengths and transferred to potato dextrose agar (PDA) medium supplemented with 60 mg/mL of streptomycin and 100 mg/mL of ampicillin using an aseptic technique to inhibit the bacterial growth. At the same time, the final sterile water used for washing the tissues (100 μL) was also plated on the PDA to confirm the sterilization effect of the surface. The inoculated plates were incubated at 28℃ in darkness for 2–15 days to allow the growth of endophytic fungal hyphae, and checked regularly. Pure isolates were checked for purity and transferred to another PDA plate by the hyphal tip method [48]. The obtained endophytic fungal isolates were coded according to their source tissues (DR1–1, DR1–2, DR2–1, etc. from roots and DS2–1, DS3–1, DS1–2, etc. from the stems). These endophytes were classified according to colony color, form, elevation and margin characteristics on PDA. Based on the groupings, strains with different morphology were screened for molecular identification.

Molecular identification and phylogenetic evaluation of endophytic fungi

According to the above simple classification, each type of fungi was chosen as the representative for molecular biological identification using the fungal genomic deoxyribonucleic acid (DNA) extraction. Fungal genomic DNA was extracted was previously described by Landum et al. according to the

manufacturer's instructions using the DNeasy Plant Minikit (Qiagen, Germany) [49]. The nuclear ribosomal DNA internal transcribed spacer (ITS) of the fungal isolates were amplified by forward primer, ITS1-F (5'-TCCGTAGGTGAACCTGCGG–3') and reverse primer, ITS4 (5'-TCCTCCGCTTATTGATATGC–3') [50]. The final reaction volume was 25 μL, containing 12.5 μL of 2X PCRBIO Taq Mix Red (PCR Biosystems, UK), 0.4 μM of forward and reverse primers and 10 ng of genomic DNA template. For negative control, the DNA was replaced with distilled water to verify absence of contamination. PCR was carried out using MyCycler ^{TM} (Bio-Rad, USA), programmed for 5 min 94°C; 30 cycles for 30s at 94°C, 60s at 55°C, and 1min at 72°C; and a final 10 min extension at 72 °C. The PCR products were separated using 1% agarose gel in 1X TAE buffer (90mM Tris-acetate and 2 nM EDTA, pH 8.0), with ethidium bromide (0.5μg/mL) staining and recorded with FluorChemTM (Alpha Innotech, USA). The PCR products were sequenced by Invitrogen Co. Shanghai.

In phylogenetic evaluation, the ITS DNA sequences and downloaded sequences of their nearest neighbors were aligned in Alignment Explorer of MEGA 4 software using ClustalW option [51, 52]. MUSCLE (UPGMA) algorithm was used to prune and verify the sequence. The evolutionary distances and history were calculated by using the neighbor-Joining methods [53]. The robustness of the trees were assessed by bootstrap analysis with 1000 replication [54].

Bioactivity evaluation

Fermentation and preparation of fungal extract

Fermentation and preparation of the fungi were determined according to the scheme proposed by Ya-Li et al. with some modifications [55]. Briefly, all isolates were cultured in potato dextrose broth (PDB, the medium contained potato 200 g and glucose 20 g in 1 L of purified water) for 14 d at 28 °C on a shaker at 180 r/min. Crude fermentation broth was filtered with eight layers of gauze. Filtered liquid was extracted three times with the same amount of ethyl acetate. The organic solvent extract was then evaporated under reduced pressure to yield an ethyl acetate extract. The ethyl acetate extracts were dissolved in methanol and the final concentration was 10 mg/mL for bioactivity screening.

Antioxidant activity

The radical scavenging ability was evaluated by using adapted 2,2'-diphenyl-b-picrylhydrazyl (DPPH) method described previously with some modification [56]. Thus, an aliquot of extract (50 μL) was added to 150 μL of methanol DPPH (50 μM). The reaction mixture was transferred to a 96-well microtitre plate and incubated at room temperature for 30 min in the dark and absorbance was measured at 517 nm using a microtiter plate reader (Bio-Rad 680, BIO-RAD, USA). Ascorbic acid (Vc) and methanol were used as positive and negative controls, respectively. Meanwhile, three experimental replicates were taken for the assay.

Antimicrobial activity

The determination of antimicrobial activity was based on the disk diffusion method with some modification [57]. Each disc (Oxford cup, 6 mm diameter) contained 200 μg of endophytic fungi extraction (10 mg/mL). The indicator organisms included gram-negative: *Escherichia coli (*ATCC25922, EC), *Pseudomonas aeruginosa*CMCC(B)10104, PA); gram-positive: *Staphylococcus aureus* (ATCC6538, SA), *Bacillus subtilis (*ATCC6633, BS); three pathogenic fungi *Penicillium* (ATCC9080, P), *Aspergillus niger (*CMCC(F)98003, AN) and*Candida albicans (*CMCC(F)98001, CA). There were purchased from Shanghai Luwei Technology Co., Ltd. Streptomycin and amphotericin B were used as positive controls and methanol as negative control. The antimicrobial activities were determined according to diameters of inhibitory zones (ZI) and experiments were repeated three times.

Anticancer activity

Human papillary thyroid carcinoma cell line IHH4 and human pancreatic adenocarcinoma cell line CFPAC–1 were obtained from the Cell Line Bank of the Chinese Academy of Science. The anticancer activity was determined according to CCK–8 assay [58]. Cisplatin was used as the positive control and repeated for three times.

Isolation of bioactive metabolites

Based on the results of the above antioxidant, antimicrobial and anticancer activities, the strain *Irpex lacteus* DR10–1 was selected for the chemical analysis because it exhibited widest broad-spectrum bioactivities. *Irpex lacteus* DR10–1 culture filtrate 14L was fermented by the same method as above mentioned. Crude ethyl acetate (EtOAc) extracts from*Irpex lacteus* DR10–1 (6.7g) was obtained and further purified by a silica gel column (200–300 mesh, 4.0 × 70 cm, with 70 g of silica gel), and eluted with gradient mixtures of petroleum ether (60–90 °C) and EtOAc to yield 5 fractions (A1-A5). Fraction A2 (156 mg) was further purified by a silica gel column chromatography (300–400 mesh, 2.0 × 25 cm, with 15 g of silica gel) and eluted with gradient mixtures of chloroform ($CHCl_3$) and EtOAc to yield compound *1.* Fraction A4 (98 mg) was further purified by a silica gel column chromatography (300–400 mesh, 1.0 × 25 cm, with 35 g of silica gel), and eluted with gradient mixtures of $CHCl_3$ and methanol (MeOH) to obtain compound *2.*

Nuclear magnetic resonance (NMR) spectra were recorded by Bruker Ascend 500 spectrometer. The spectrometer operated at 500 MHz for ^{1}H nuclei and 125 MHz for ^{13}C nuclei. Chemical shift was quoted in parts per million (ppm), referring to the appropriate residual solvent peak.

Statistical Analysis

Using species as the statistical unit, the number of isolates (*N)* and the isolation frequency (*IF)* for each endophytic fungal species in different tissues or the total plant (Table S1) were calculated. Species

richness index (*S)* and Margalef index (*D´)* were used to evaluate species richness, which were two important parameters for alpha diversity analysis [59]. Shannon-Wiener index (*H´)* and Simpson's diversity index (*D*s) were used to the species diversity, respectively [60, 61]. Additionally, the Jaccard Similarity Index (*JC)* was used to compare the species composition of the stem and root tissues [62]. Results were expressed as mean ± standard deviation (SD) of triplicate of measurements for the DPPH and CCK–8 assays. Data were conducted with SPSS 18.0 for Windows (SPSS Inc., Chicago, USA).

Results

Community composition and abundance

A total of 154 fungal endophytes were isolated from *D. chinense* plants collected from the TGR area. Among them, 30 different representative morphospecies were determined according to cultural characteristics (Fig. 1B). Of these detected, 30 isolates were categorized into 27 different taxa (Ascomycota, 19; Basidiomycota, 8), and further into nine distinct orders (Fig. 1C). The Fig. 2 showed the phylogenetic tree of 30 fungal strains isolated from the NCBI database and the accession numbers of the matched rDNA-ITS sequences. The supplementary table data (Table S1) provided detailed information on 30 representative strains, including their sources and isolation frequencies.

At the order level, the Diaporthales possessed the most taxa, six taxa, accounting for 22.2% of the total fungal taxa and they had 48 isolates, around 31.2% of the total fungal isolates (Fig. 1D). Conversely, the Botryospaeriales had the most isolates, 52 isolates, accounting for 33.8% of the total fungal isolates and they possessed four species, around 14.8% of the total fungal species. The Polyporales and Agaricales were the second and third most abundant orders with high species, and together constituted approximately 33.3% of all the species. Analogously, the Xylariales and Polyporales were the second and third most abundant isolates, and together constituted approximately 18.1% of all the isolates. The other identified orders were the Hypocreales, Microascales, Eurotiales and Discellaceae, which together constituted approximately 22.2% and 10.4% of all species and isolates, respectively (Fig. 1D). Interestingly, the most common fungal species between roots and stems were *Phomopsis* sp. (24.7%), followed by *Neofusicoccum parvum* (23.4%). However, *Phomopsis* were not from order with highest isolate rates.

Species diversity and richness abundance of fungi

The richness and species diversity of culturable endophytic fungi were significantly higher in stems than in roots (Table 1). Among the 27 total taxa, 16 (59.3% of total) were obtained from the stems. A total of 3 fungal taxa- *Neofusicoccum parvum, Phomopsis* sp. and *Diaporthe* sp. were distributed in both plant tissues, but ten taxa-*Fusarium* sp., *Fusarium equiseti, Xylaria venosula, Lasiodiplodia theobromae, Penicillium ochrochloron, Rhizoctonnia bataticola, Robillarda sessilis, Coprinellus xanthothrix, Polyporus crassa* and *Irpex lacteus* were only found in the roots (Fig. 3). Similarly, of the nine orders, two were found in both stems and roots, but the Hypocreales, Xylariales, Eurotiales and Discellaceae were unique to the

roots (Fig. 4). Additionally, Shannon-Wiener index (*H')* and Simpson diversity index (*Ds)* can be used to analyze species diversity, and the species richness (*S)* and Margalef index (*D')* can reflect the richness of endophytic fungi species. As shown in Table 1, the species richness and diversity of endophytic fungi in stems were higher than those in roots, and the values of *S* (16), *D' (*3.5802), *H'* (2.5323) and *D*s (0.8659) were higher. In addition, the similarity index (Jaccard's index) was used to estimate the similarity between stem and root. Although stem and root samples collected in TRG field were adjacent to each other and lived in the same place, the Jaccard's index only showed 0.11 between stems and roots, showing low similarity. These indices showed that endophytic fungi in different tissues had significant diversity.

Bioactivity evaluation of fungal endophytes

As mentioned above, one of the main purposes of this study was to identify endophytic fungi that could be cultured and applied to develop their potentially beneficial properties for plants and humans. All 154 fungal endophytes isolated from *D. chinense* at TGR were evaluated for their antioxidant, antimicrobial and anticancer activities (Table S2-S4). Among the 154 isolates, 99 (64.3%), 153 (99.4%) and 27 (17.5%) fungal extracts showed antioxidant activity, antimicrobial activity against at least one indicator organisms and anticancer activity against one or two human cancer cell lines, respectively. Among the isolates that displayed the individual activities, *Phomopsis* sp. accounted for 20, 38 and 4 of isolates possessing antioxidant, antimicrobial and anticancer activities, respectively (Fig. 5). *Neofusicoccum parvum* and *Xylaria venosula* were also enriched in isolates showing bioactivities. However, in our assay, the isolates belonging to *Mycorrhiza basidiomycete,* did not display antioxidant and anticancer activities (Fig. 5). Thus, the distribution of active strains showed obvious taxonomic specificity. Interestingly, the fungal extracts of DS16–1 (*Phomopsis* sp.), DR10–1 (*Irpex lacteus),*, DS9–1 (*Periconia* sp.) and DS6 (*Phomopsis* sp.) showed higher antioxidant activity than that of ascorbic acid, acted as a scavenger of DPPH radical with IC_{50} values of 2.59 ± 0.03, 2.79 ± 0.04, 2.95 ± 0.03 and 2.97 ± 0.01 μg/mL, respectively. For the antimicrobial activity, fungal extract of DR28–1 (*Phomopsis* sp.) displayed the highest antimicrobial activity against *Pseudomonas aeruginosa* with a zone of inhibition (ZI) value of 40 mm, fungal extract of DS35–1 (*Ceriporia lacerta)* showed the highest antimicrobial activity against *Staphylococcus aureus,* with a ZI value of 40 mm, the fungal extracts of DR41–2 (*Ceriporia lacerta)* had the highest activity against *Aspergillus niger,* and its ZI value was 30 mm. Particularly, the extract DR10–1 (*Irpex lacteus)* was the only strain that exhibited broad antimicrobial capability because it inhibited the growth of all tested pathogens. As for anticancer activity, fungal extract of DR46–1 (*Phomopsis* sp.) showed the highest anticancer activity against IHH4 cell line with IC_{50} values of 9.20 ± 0.02 μg/mL.

Characterization of metabolites of strain DR10–1

According to the bioactivity screening results of the 154 strains of endophytic fungi extracts, we discovered that strain DR10–1(*I*rpex *lacteus)* showed higher antioxidant activity than that of ascorbic acid, displayed antimicrobial capability by inhibiting the growth of seven tested pathogens and showed

anticancer activity against both tested cancer cell lines. In order to specifically study the chemical components of the only broad-spectrum active DR10–1 strain, large-scale fermentation was carried out using the same method as mentioned above. As a result, two known metabolites were obtained which were indole–3-carboxylic acid [63] (*1)* and indole–3-carboxaldehyde [64] (*2)*(Fig. 6).

Discussion

Although *D. chinense* is a waterlogging-resistant medicinal plant, its endophytic community is rarely known. Considering the new roles of endophytic fungi in plant development, growth, adaptability and diversity, we need to fill this gap in order to exploit of endophytes for a better understanding of *D. chinense* plant and its important metabolites found in the TGR. Therefore, one of the purposes of this study was to examine the community composition of fungal endophytes from TGR. To our knowledge, our study is the first report on isolation and identification of fungal endophytes. Here, we took a culture-dependent approach, since our final goal is to build a working collection of fungal endophytes that can be explored for their potentially beneficial properties in *D. chinense* plant. In this work, a total of 154 endophytic fungi were isolated from D. chinense in the TGR and classified into 27 different taxa according to their morphological characteristics and unique phenotypic characters. Among them, the fungi that belong to Phomopsis, Diaporthe, Fusarium and Irpex, have been reported as the main endophytes of wetland shrub Myricaria laxiflora in the TGR [65] and riparian plant species [66]. This is also in accordance with the report that Fusarium, Phomopsis and Irpex are not sensitive to flooding stress [65]. Previously, it has been reported that the assembly of land plant endophytic fungi is composed of representatives Sordariomycetes, Dothideomycetes and Pezizomycetes [67, 68], while plants from water or moist environments are more often parasitized by Eurotiomycetes [69]. In the current study, Sordariomycetes was the most prevalent class with relative frequency of 50%, while Dothideomycetes and Eurotiomycetes had a relative frequency of 33.8% and 1.3%, respectively. Thus, our data indicated that both terrestrial and aquatic fungi are present in the *D. chinense* plant. Our results showed similarity to those of Kandalepas *et al.,*, who discovered high numbers of Sordariomycetes and low numbers of Dothideomycetes and Eurotiomycetes in wetland plants from Louisiana [66]. Additionally, out of 27 taxa detected, 7 taxa were darkly pigmented with thickly-walled septate hyphae that belongs to *Diaporthales, Phomopsis* sp., *Lasiodiplodia theobromae, Neofusicoccum parvum,Irpex lacteus, Periconia* sp., *Botryosphaeria dothidea,* which were referred to as dark septate fungi (DSE) [70]. Among the 154 isolates, 20 (13.0%) belongs to this group. The result showed that *D. chinense* were colonized by abundant DSEs, as some other researchers reported their occurrence in wetland plant species [71, 72]. Jumpponen and Trappe suggested that DSE frequently play unique roles in terrestrial ecosystems [73]. Therefore, these special endophytic fungal communities not only revealed the apparent environmental specificity of the TGR area, but also helped to understand the special ecological functions shown by these fungal group.

Interestingly, many isolates from the genera *Phomopsis, Fusarium, Diaporthe, Neofusicoccum parvum, Xylaria venosula, Lasiodiplodia theobromae* and *Botryosphaeria dothidea* were common and well-known pathogens but also common endophytes existing asymptomatically [74, 75]. Among them, *Diaporthe* and *Phomopsis* complex are the causes of seed decay and cause soybean blight and canker diseases [76].

Neofusicoccum parvum was reported as one of the most aggressive causal agents of the trunk disease Botryosphaeria dieback [77]. *Botryosphaeria* and its anamorph complex are particularly important for symptoms such as fruit rot, shoot blight, dieback and canker of numerous woody hosts [78]. However, it is incredible that symptoms of the disease did not appear in plants collected by *D. chinense.* This phenomenon suggests that fungal species may represent an evolutionary transition, or simply fungi have achieved remarkable ecological plasticity, thus ensuring the optimal growth and reproduction of various hosts, which ultimately leads to the expansion of their bio-geographic distribution [79]. On the other hand, most of the transitions from a mutualistic to a parasitic interaction are characterized by imbalance in nutrient exchange [80] or by environmental variations [81, 82]. Furthermore, if plants are under physiological stress, the type of interaction between endophytes and host plants are also regulated [83]. In this regard, the mutualistic interactions between fungal invaders and host plants are deciphered as a balance, which is considered as a combination of environmental and physiological effects that benefit both sides [80]. The adaptive benefits of mutualistic fungi promote or are responsible for plant adaptation to biotic and abiotic stress by increasing resistance to drought and water stress [84, 85]. In addition, several other genera were also isolated from *D. chienense,* including *Penicillium ochrochloron, Mycorrhizal basidiomycete, Ceriporia lacerta, Diaporthe longicolla, Diaporthe eres, Flavodon flavus, Irpex* sp., *Parphoma* sp. and *Phoma medicaginis.* Although they were obtained with low relative abundant, those minor genera may play an important ecological role in their host plants, or may be able to synthesize bioactive compounds [86]. Therefore, fungal isolates reported here may have a positive impact on the health of *D. chinense* by maintaining a balance in the composition of the associated microbiome, serving as a defense or helping to deal with water stress.

Another objective of this study was to assess the potentially beneficial properties of endophytic fungi to humans. All the endophytes extracts were screened for antioxidant, antimicrobial and anticancer activities and they showed at least one biological activity. Among the screened isolates, 99 (64.3%) isolates exhibited remarkable antioxidant activity, of which 18 (11.7%) had very notable activity with IC_{50} value of ≤ 3 μg/mL, suggesting that it may protect D. chinense from oxidative stress in the flooding environment as suggested by Zeng et al [87]. Because of the protective effect of antioxidants, they are essential for plant survival and fitness and presumably selection have leaded to both redundant and highly specific pathways that address ROS production and stress mediation [88]. For example, Mirzahosseini et al. have reported that endophytic fungi can alleviate the oxidative damage produced by ROS accumulation in plant cells such as *F. arundinacea [*89, 90]. Regarding antimicrobial activity, 31.2%, 11.7%, 19.5%, 69.5% and 29.9% extracts of endophytes showed activity against *Penicillium, Candida albicans, Aspergillus niger, Staphylococcus aureus* and *Escherichia coli* respectively, which was comparable and even exceeded some results reported by other authors in similar studies [91, 92]. For example, from the 39 endophytic fungal extracts of *Viguiera arenaria* and *Tithonia Diversifolia* plants, Guimaraes et al. found only 5.1% and 25.6% extracts to be active against *Staphylococcus aureus* and *Escherichia coli* respectively [93]. Unexpectedly, *Pseudomonas aeruginosa* was most sensitive to the fungal extracts among the tested bacterial though it was reported to be drug resistant towards many antibiotics [94]. Usually, the fungal extracts also showed higher activity against the Gram-negative than

the Gram-positive ones. This different sensitivity has been suggested to be attributed to the high level of lipopolysaccharides that are contained in the Gram-positive bacteria membrane, which could make the cell wall impermeable to bioactive compounds [95]. As for anticancer activity, 27 out of 154 fungal exacts (17.5%) showed activity against IHH4/CFPAC–1 cell line, in which 11 fungal extracts were active against both tested celllines. Statistically, 18 out of 27 anticancer isolates were exclusively isolated from the roots, 9 were only recovered from stems. Generally, for the same fungal species e.g. Neofusicoccum parvum, the isolates from roots showed stronger bioactivity compared to those from the stems regardless of antimicrobial, antioxidant or anticancer bioactivities. Such data well supported the traditional practice of native people who often used the extracts from roots to relieve analgesic, antirheumatic and diuretic [43].

Of these isolates screened, a high proportion of bioactivities were mostly detected from the fungal extracts belonging to *Phomopsis* sp. (24.7%), *Neofusicoccum parvum* (23.4%) and *Xylaria venosula* (9.1%), which was attributed to their high separation rate. As did here, *Phomopsis* sp. have been reported as dominant member of the endophytic community [96]. *Phomopsis* is a dominant member of the endophytic community because it grows rapidly, thus inhibiting slow growing endophytes, which might be one of the reasons for the low number of species detected in this study [97]. Additionally, *Phomopsis* and related taxa contain important endophytic and are known to produce a series of bioactive secondary metabolites in vitro with a variety of different chemical structures [98]. However, few studies conducted on the active metabolites of *Neofusicoccum parvum,* and its antioxidant activity accounted for the highest proportion in the current study, which has never been reported in previous studies [99, 100]. Besides, *Xylaria* species are widely distributed on the temperate to the tropical zones in the terrestrial globe, and fungi of this genus have been proved to be potential sources of novel secondary metabolites, and many of them have biological activities related to drug discovery, including cytotoxic, antimalarial, and antimicrobial activities [101]. In terms of bioactivity, active extracts of DS16–1 (*Phomopsis* sp.), DR28–1 (*Phomopsis* sp.), DS35–1 (*Ceriporia lacerata),,* DR41–2 (*Ceriporia lacerata)* and R46–1 (*Phomopsis* sp.) were found promising. In particular, the strain DR10–1(*Irpex lacteus)* showed wide spectrum bioactivities, suggesting that possible use of one endophyte could be a valuablecandidate as new antioxidant, antimicrobial and anticancer agents.

Finally, we isolated two known compounds including indole–3-carboxylic acid and indole–3-carboxylic acid derivatives from the wide spectrum bioactive strain *Irpex lacteus* DR10–1. As far as we know, this is the first time that indole–3-carboxylic acid (*1)* and indole–3-carboxaldehyde (*2)* have been isolated from endophytic fungus *Irpex lacteus.* It was previously demonstrated that indole–3-carboxylic acid isolated from endophytic fungal strain of Epicoccum nigrum associated with Entada abyssinica had remarkable activity against Gram-negative strains (Staphylococcus aureus) with MIC values of 6.25 μg/mL [102]. This finding is consistent with literature report on the antibacterial activity of indole–3-carboxylic acid, from which a novel series of indole–3-carboxylic acid derivatives were previously reported to possess potent antibacterial activity against *Enterococcus faecalis [*103]. In addition, it has been reported that indole–3-carboxylic acid had weak cytotoxic effects on both normal and tumor cells, and its antioxidant activity is weak [102]. Recently, the indole–3-carboxylic acid (IAA) and other auxins have been shown to

stimulate cell elongation, resulting in root growth initiation or an enhancement of nutritional elements absorption by the hosts [104, 105]. Besides, IAA was supposed to improve the adaptability of plant microbe interaction [106].

Conclusions

Taken as a whole, we analyzed the fungal endophytes of D. chinense extensively and concluded that D. chinense harbored diverse fungal species. Most of these fungal endophytes showed bioactivity including antioxidant, antimicrobial and anticancer activities. Specifically, the widest broad-spectrum bioactive DR10–1 strain from D. chinense produced several active compounds, which played crucial roles in plant growth promotion and human medicinal value. The fungal endophyte of DR10–1 is a good resource for obtaining novel drugs. Therefore, this study provides a framework for further research and utilization of endophytic fungi as a unique source of interesting and useful bioactive compound.

Declarations

Abbreviations

TGR: Three Gorges Reservoir; ROS: Reactive Oxygen Species; HgCl: Mercuric Chloride; PDA: Potato Dextrose Agar; DNA: DeoxyriboNucleic Acid; ITS: Internal Transcribed Spacer; PDB: Potato Dextrose Broth; DPPH: 2,2'-diphenyl-b-picrylhydrazyl; Vc: Ascorbic Acid; EC: *Escherichia coli;*PA: *Pseudomonas aeruginosa;*SA: *Staphylococcus aureus;*BS: *Bacillus subtilis;*P: *Penicillium;* AN: *Aspergillus niger;*CA: *Candida albicans;*ZI: Inhibitory Zones; EtOAc: Ethyl Acetate; $CHCl_3$: Chloroform; MeOH: Methanol; NMR: Nuclear Magnetic Resonance; *N:*Number of Isolates; *IF:*Isolation Frequency; *S:*Species Richness Index; *D´:* Margalef Index; *H´:*Shannon-Wiener Index; *D*s: Simpson's Diversity Index; *JC:* Jaccard Similarity Index; SD: Standard Deviation.

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Author Contributions

X. X. contributed to the experimental conception and design, the process of the whole project for carrying out the related experiments, data analysis, and manuscript draft; F. F. contributed to the fungal isolate culture and data analysis; D. Q., T. C., W. Y., S. H., B. H., J. R., Y. J., contributed reagents, materials, and analysis tools; J. Y. contributed to the experimental design as well as the manuscript draft and improvement.All authors read and approved the final manuscript.

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Supplementary Materials

Supplementary materials can be found in this paper.

Ethics approval and consent to participate

No specific permission was required for the described study area. The research work doesn't involve any endangered or protected plant species.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Tables

Table 1. Diversity analyses of endophytic fungi from *D. chinense*

Diversity Index	Different Tissues		Total
	Root	Stem	
Species richness (*S*)	14	16	27
Margalef index (*D´*)	2.9109	3.5802	5.1619
Shannon-Wiener index (*H´*)	2.1828	2.5323	2.4824
Simpson diversity index (*Ds*)	0.8366	0.8659	0.8646
Jaccard's indice (*JC*)		0.11	

Figures

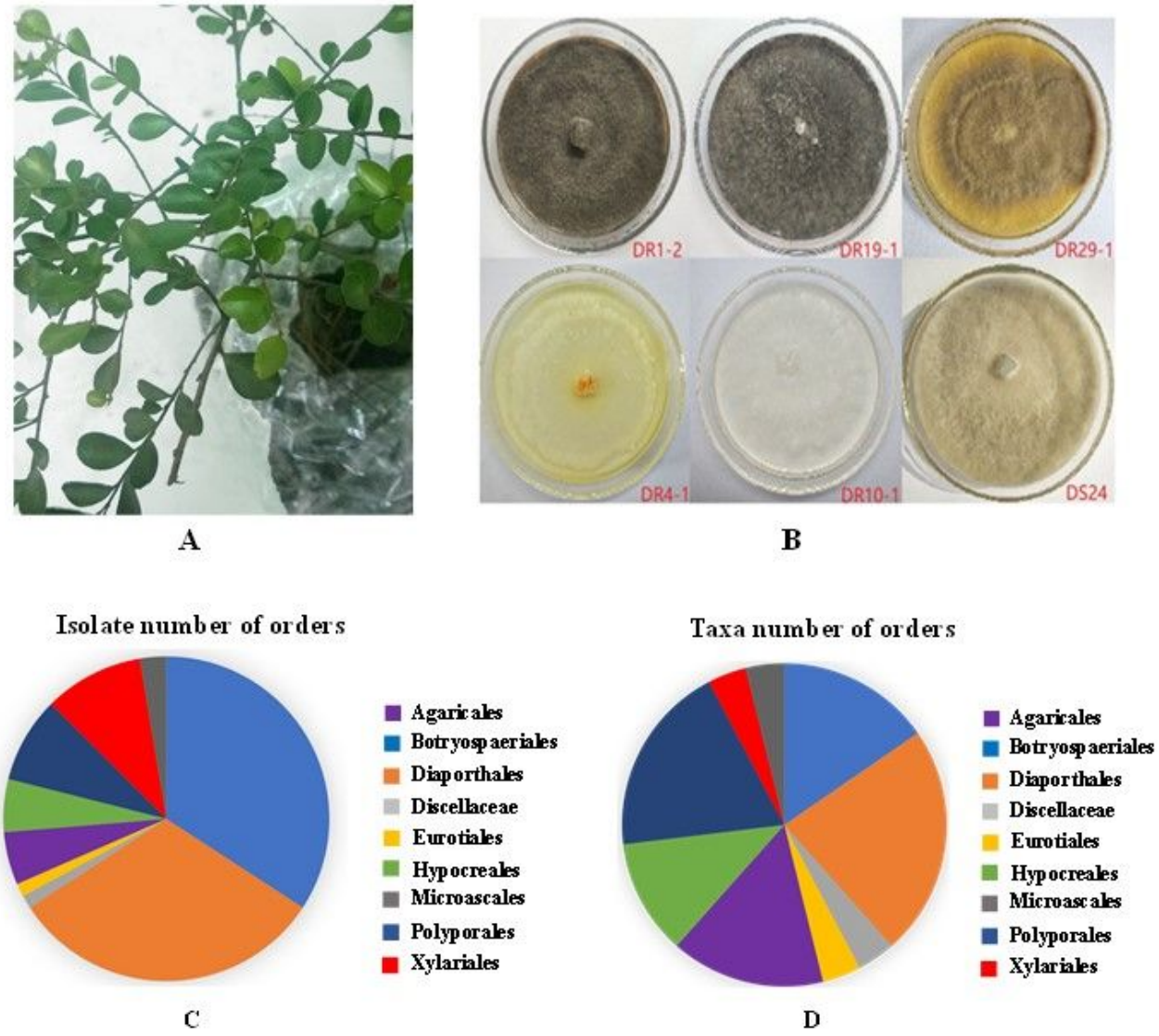

Figure 1

. D. chinense plant and taxonomic distribution of endophytic fungi. (A) D. chinense plant. (B) Representative fungal morphotypes isolated from D. chinense growing on potato dextrose agar (PDA) for one week at 26 ℃. (C) Distribution of fungal isolates (n=154) belonging to each order (n=9). (D) Distribution of fungal taxa (n=27) belonging to each order (n=9).

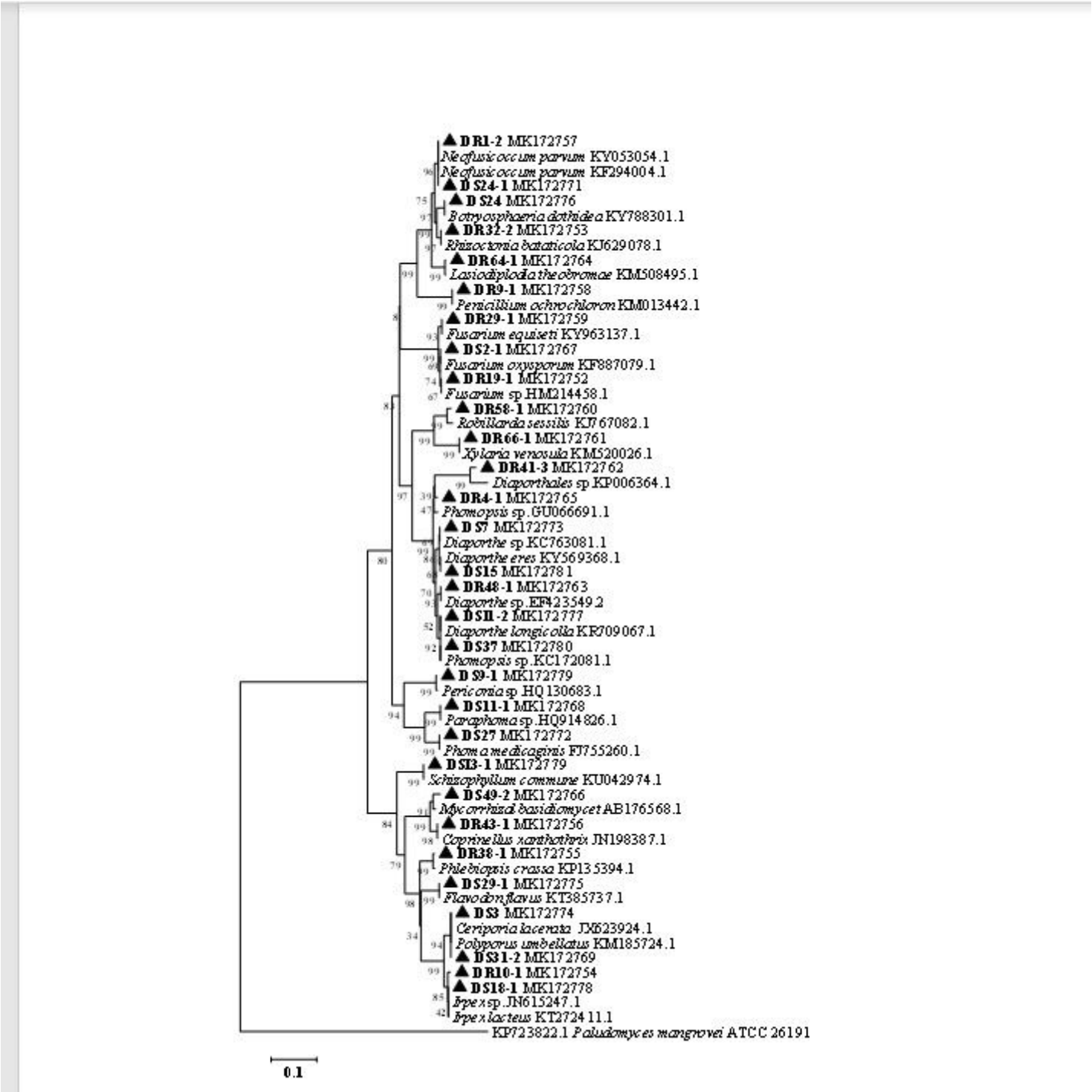

Figure 2

Phylogeny analyses of endophytic fungi from D. chinense. The tree was derived by neighbor-joining methods analysis of ITS1-5.8S-ITS4 sequences [53] and 30 sequences retrieved from Gen Bank. The percentage of replicate trees in which associated taxa were clustered together in the bootstrap test (1000

replicates, values below 50% are not shown) are shown next to the branches. Phylogeny analyses were conducted in MEGA 4 software [51, 52].

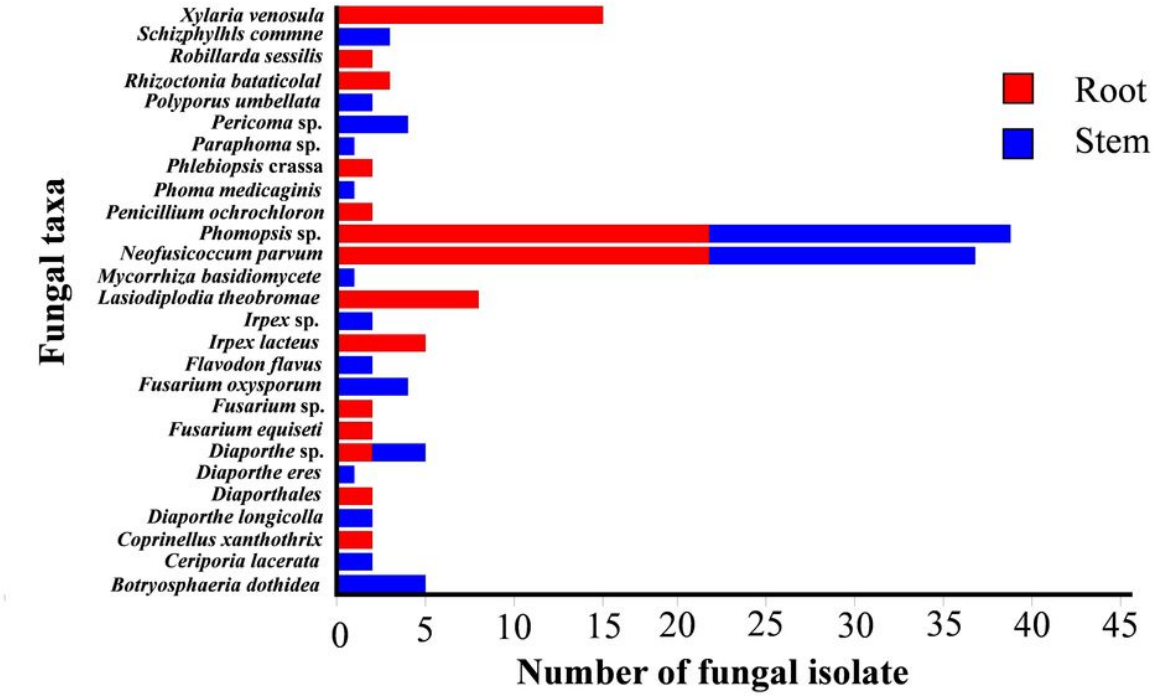

Figure 3

Distribution of the fungal isolates (n=154) across different plant tissues.

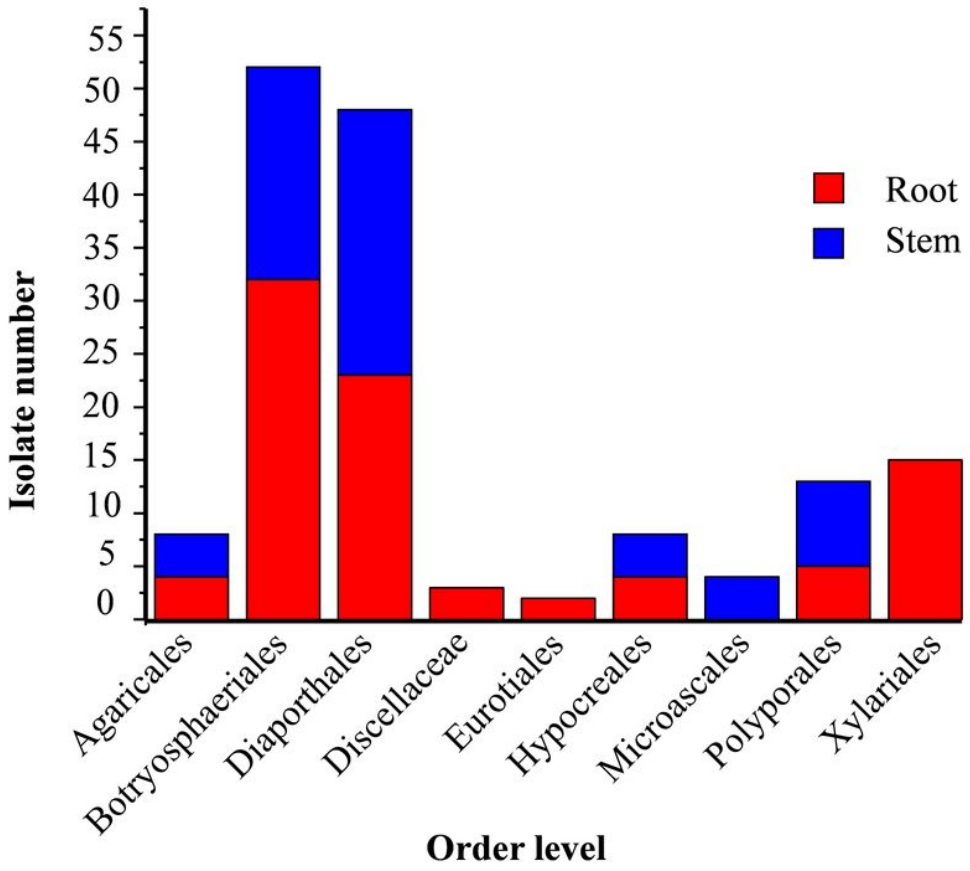

Figure 4

Distribution of the orders of the fungal isolates (n=154) from different tissues.

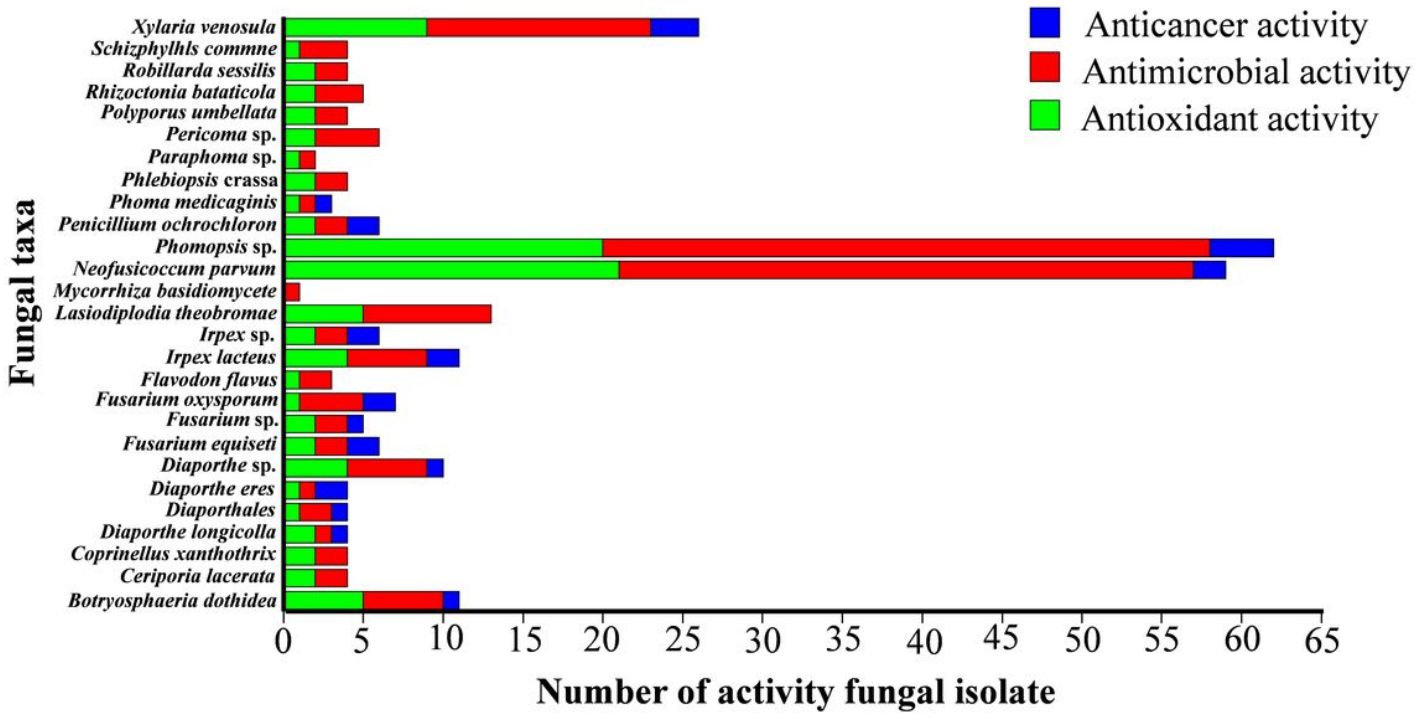

Figure 5

Distribution of the activity fungal isolates.

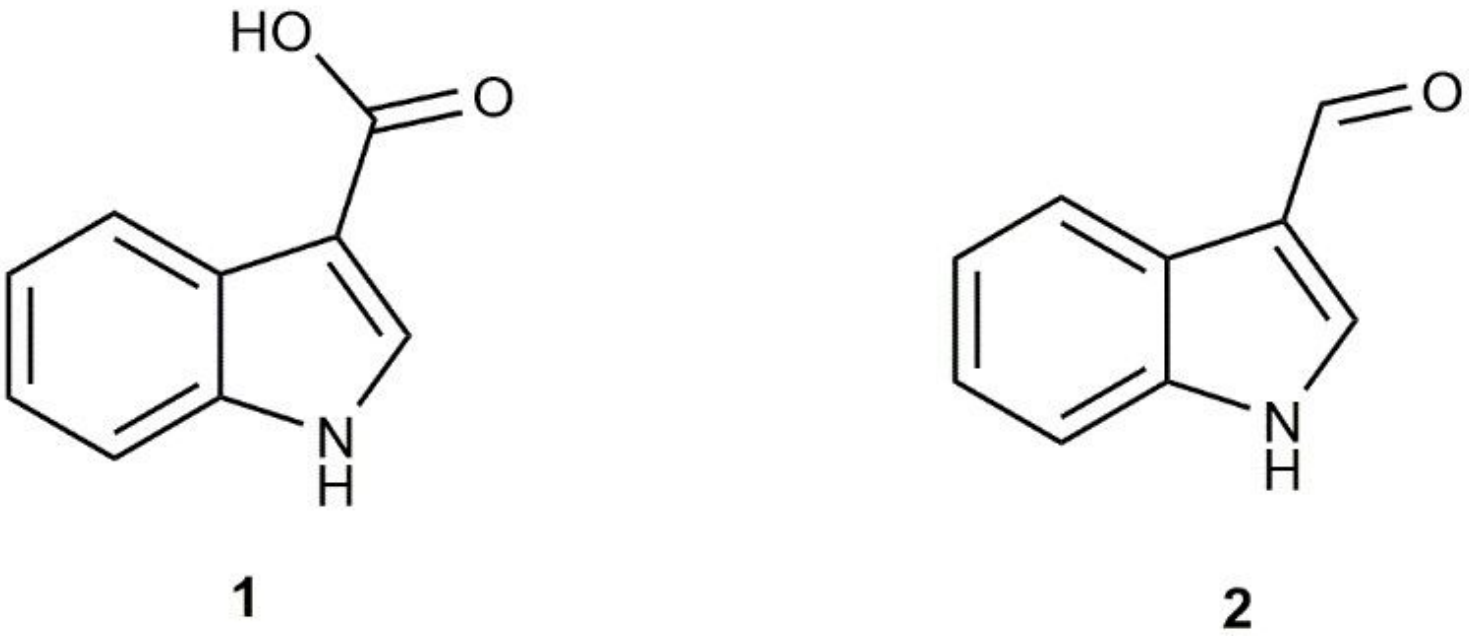

Figure 6

The chemical structure of compounds 1-2.

Supplementary Files

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- Supplementarymaterial.docx