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Fungal endophytes of *Taxus* species and regulatory effect of two strains on taxol synthesis

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Abstract

Background Taxol, derived from *Taxus* trees, is a valuable natural resource for the development of anticancer drugs. Endophytic fungi from *Taxus* trees are a promising alternative source of Taxol. However, the impact of plant-endophytic microbial interaction on the host's Taxol biosynthesis is largely unknown.

Results In the current study, the diversity of endophytic fungi in three different *Taxus* species was analyzed using Internal Transcribed Spacer sequencing. A total of 271 Operational Taxonomic Units (OTUs) were identified, grouping into 2 phyla, 8 classes, 16 orders, 19 families, and 19 genera. Alpha and beta diversity analysis indicated significant differences in endophytic fungal communities among the various *Taxus* trees. At the genus level, *Alternaria* and *Davidiella* were predominantly found in *T. mairei* and *T. media*, respectively. By utilizing a previously published dataset, a Pearson correlation analysis was conducted to predict the taxol biosynthesis-related fungal genera. Following screening, two isolates of *Alternaria* (L7 and M14) were obtained. Effect of inoculation with *Alternaria* isolates on the gene expression and metabolite accumulation of *T. mairei* was determined by transcriptomic and untargeted metabolomic studies. The co-inoculation assay suggests that the two *Alternaria* isolates may have a negative regulatory effect on taxol biosynthesis by influencing hormone signaling pathways.

Conclusion Our findings will serve as a foundation for advancing the production and utilization of *Taxus* and will also aid in screening endophytic fungi related to taxol production.

Keywords Correlation analysis, Diversity, Endophytic fungi, ITS sequencing, *Taxus*

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Introduction

Taxus species are widely distributed in the world [1, 2]. In China, most of the *Taxus* species have been included in the national protection scope for rare and endangered plants [3, 4]. *Taxus* plants adapt to a variety of changing environments, from cold zones to temperate zones across a latitudinal gradient in China [5]. The conservation of endangered *Taxus* trees and their biodiversity, including population size, geographical distribution, and habitat, is an urgent priority [6].

As medicinal plants, *Taxus* contain natural anticancer components, such as taxol (generic name paclitaxel) and its precursors, which have high medicinal value [7, 8]. Modern medical research confirms that taxol can be used to treat a variety of cancers, including ovarian, lung, cervical, and esophageal cancers [9]. Due to its low content in *Taxus* tissue, the significant medicinal value of paclitaxel keeps its price high [10].

Endophytic fungi, which are widely present in plant tissues, play a crucial role in plant growth, development, and secondary metabolism, typically without causing any damage to the plant itself [11, 12]. The relationship can vary from latent pathogens or saprotrophs to mutualism, and the fungi can be facultative endophytes. In most cases, endophytic fungi contribute to plant survival by promoting plant growth, inhibiting competitors, and enhancing stress resistance [13, 14]. In addition, some endophytic fungi produce compounds similar to those produced by their host plants. For instance, Taxol was identified and isolated from endophytic fungi found in *T. brevifolium* [15]. Over time, numerous taxol-producing fungi have been isolated from various *Taxus* species, such as *Aspergillus fumigatus* from *T. wallichiana*, TMS-26 from *T. media*, *Cladosporium* from Iranian *T. baccata*, and *Paraconiothyrium* sp. from English *T. baccata* [16–18]. However, recent studies have challenged the idea of taxol production by endophyte species, as the biosynthetic gene cluster has never been discovered in endophytes [19]. Throughout our research, we have not yet identified a fungus capable of producing taxol. Therefore, our study focuses on screening fungi that can influence the synthesis of taxol in *Taxus*, rather than those that can directly produce taxol. A comprehensive analysis of endophytic fungi in *Taxus* plants is essential for identifying novel taxol biosynthesis-related fungi.

The culture-dependent method is widely used to determine the diversity of endophytic fungi [20]. However, due to limitations in microbial culture technology, isolating and culturing all endophytic fungi in Taxus trees is challenging. High-throughput sequencing plays a crucial role in exploring the diversity of endophytic fungi, providing comprehensive information about these fungi in different *Taxus* species [21]. To date, the diversity of endophytic fungi has been investigated in various *Taxus* species. Molecular phylogenetic analysis identified 245 endophytes in *T. globose* [22]. Morphological and molecular identification isolated 81 endophytic fungi from *T. media* [23]. In the Himalayan yew (*Taxus wallichiana*), 13 distinct endophytic fungi were identified using the Internal Transcribed Spacer (ITS)-PCR method [24]. A total of 115 endophytic fungi isolates were isolated from the bark of *T. chinensis* by ITS1-5.8 S-ITS2 sequencing method [25]. In the Iranian yew (*T. baccata*), 125 fungal strains from 21 genera and 26 species were isolated and identified [26]. However, the variations in endophytic fungal communities among different *Taxus* species remain largely unknown.

A recent report revealed that fungal elicitors could significantly enhance taxol biosynthesis in *Corylus avellana* cell culture [27]. To date, several endophytic fungi from *Taxus* trees have been reported to be involved in Taxol biosynthesis. For example, *Aspergillus niger* was isolated from the inner bark of *T. chinensis*, and *Pseudodidymocyrtis lobariellae* was isolated from the needles of *T. chinensis* as fungal elicitors [28, 29]. *Taxomyces andreanae*, a fungal endophyte isolated from the Yew tree *Taxus brevifolia*, is another alleged taxol-producing fungus [30]. Screening for additional Taxol biosynthesis-related endophytes is an environmentally friendly approach to enhance the mass production of Taxol.

In the current investigation, three *Taxus* species were studied, including *T. media*, a natural hybrid known for its high taxol content, *T. mairei*, found in the Yangtze River Basin of China, and *T. cuspidata*, primarily located in Changbai Mountain. The study aimed analyze the differences in endophytic fungi communities [31, 32]. Through Pearson correlation analysis, several taxol biosynthesis-related fungal genera were identified. Furthermore, effect of inoculation with *Alternaria* isolates on the gene expression and metabolite accumulation of *T. mairei* was determined by transcriptomic and untargeted metabolomic studies. These findings offer crucial foundational information for the holistic utilization of *Taxus* and aid in the identification of taxol production-related endophytic fungi.

Materials and methods

Plant samples and DNA extractions

Barks were collected from five-year-old cultivated *Taxus* plants. *T. media* trees were obtained from Quzhou City, China, *T. mairei* plants were obtained from Yaan City, China, and *T. cuspidata* from Jilin City, China. All plants were kept in a growth chamber at Hangzhou Normal University, Hangzhou, China, at 28±2 °C with a light/dark cycle of 14/8 h and a 65–70% relative humidity for one month. Total DNAs from the twigs of different *Taxus* trees were isolated using a Fast-DNA SPIN extraction kit (MP Biomedicals, Santa Ana, CA, USA) according to the

kit's instruction. Doble Distilled H_2O (ddH_2O) was used as a negative control. Total DNAs were eluted in 50 µL of ddH_2O and stored at -80 °C until used. For each *Taxus* tree, fifteen biological replicates were performed for ITS sequencing.

PCR amplification and ITS sequencing

The ITS region of the eukaryotic small-subunit rRNA gene was amplified with primers ITS7 (5'-GTGARTCATCGAATCTTTG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [33]. Specific barcodes were connected to the 5' end of ITS4/7 primers. PCR amplification was carried out in a reaction mixture solution containing 25 ng of template DNAs, 12.5 µL of PCR Premix, 2.5 µL of each primer, and ddH_2O to adjust to a 25 µL final volume. The PCR condition to amplify the ITS fragments consisted of an initial denaturation at 98℃ for 30 s; 35 cycles of denaturation at 98 ℃ for 10 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 45 s; and final extension at 72℃ for 10 min. The PCR products were checked by 2% agarose gel electrophoresis. The purified PCR products were quantified using the Qubit kit (Invitrogen, USA). The DNA library for sequencing was prepared on an Agilent 2100 Bioanalyzer (Agilent, USA) with a Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA). The resulting libraries were sequenced using the 250PE MiSeq platform provided by LC-Bio (Hangzhou, China).

OTU and diversity analysis

Raw reads were isolated based on their unique barcodes. After removing the barcode and primer sequence, clean reads were quality filtered using FastQC software (v.0.10.1). Chimeric sequences with ≥97% similarity were produced and assigned to specific operational taxonomic units (OTUs) by Verseach (v.2.3.4). The representative sequence for each OTU was then assigned to each representative sequence using the Ribosomal Database Project classifier program.

OTUs abundance levels were normalized, and multiple sequence alignment analysis was performed using PyNAST software [34]. Alpha diversity parameters, such as Chao1, Shannon, Simpson, and Observed species, were utilized to analyze the complexity of species diversity. These were calculated using QIIME (v. 1.8.0). Beta diversity analysis was conducted to assess differences in species complexity among samples. This analysis was performed through principal co-ordinates analysis (PCoA) and QIIME software (Version 1.8.0), respectively.

Phylogenetic study

An unrooted phylogenetic tree was constructed using MEGA 6.1 (http://www.megasoftware.net/) employing the neighbor-joining method. The values marked on the branch of the phylogenetic tree mainly represent the support or reliability of the branch. To quantify the effectiveness of the branch, the values are calculated with bootstrap method.

Pearson correlation coefficient

The correlation between taxoids and OTUs was made with the Pearson correlation coefficient using the 'corrplot' package available in OmicStudio (https://www.omicstudio.cn/home). Pearson's correlation coefficient cut-off of 0.6 and P value <0.05 were stablished to consider significant and strong correlations.

Isolation and characterization of endophytic fungal isolates

Fresh leaves of *T. mairei* were surface washed and sterilized with 75% ethanol and 1.5% sodium hypochlorite washing solution twice. The clean samples were cut into pieces and placed on solid Potato Dextrose Agar (PDA) medium supplemented with 30 µg/mL streptomycin sulfate. The emerging fungal colonies were harvested and isolated by plate streaking. The genomic DNA of each fungal isolate was extracted using the FirePureTM Microbial DNA Extraction Kit (FG0308, FIREGEN). The ITS region was amplified using ITS4/7 primers. The PCR products were recovered, sequenced, and searched against the NCBI GenBank database to identify fungal species and genera (**Table S1**).

Inoculation of endophytic fungi with *T. mairei*leaves

The clean leaves of *T. mairei* were collected and sprayed with 1 mL of spore suspension containing the two endophytic fungal isolates (L7 and M14). The concentration of the spore suspension was adjusted to 1×10^6 spores/mL. ddH_2O was used as a control. After 48 h incubation, the treated leaves were cleaned and harvested for transcriptomic and metabolic analyses.

RNA isolation and RNA-sequencing

Total RNAs of *T. mairei* leaves were extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The purified and fragmented mRNAs were used to construct cDNA libraries using a VAHTS Universal V6 RNA-seq Library Prep Kit according to the manufacturer's instructions. RNA sequencing was conducted on an Illumina 6000 platform (OEbiotech, Shanghai, China) to produce raw sequences according to the manufacturer's protocol.

Analysis of the transcriptomic data

Raw fastq format reads were processed to produce clean reads using fastp program. The clean reads for each sample group were mapped onto the *T. mairei* genome using HISAT2 software [8]. Fragment Per Kilobase of transcript per Million mapped reads (FPKM) method was applied to

calculate the read counts of each gene by HTSeq-count. Differentially expressed genes were screened using the DESeq2 software based on a threshold of P value < 0.05 and fold change > 2 or fold change < 0.5. Most of the differentially expressed genes (DEGs) were classified into different Gene Ontology (GO) terms and/or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways by the Blast2GO program (https://www.blast2go.com/). For the enrichment analysis, a GO or KEGG group with a corrected P value < 0.05 was considered significant.

Untargeted metabolomic analysis

The separation was carried out on a 100×2.1 mm, 1.7-μmparticle size Waters ACQUITY UPLC BEH C18 column using an UPLC system (Waters, Herts, UK). Ahigh resolution MS/MS Waters Xevo G2-XS Q-TOF (Waters, Herts, UK) was used to detect metabolites eluted from the column. Raw data of LC-MS/MS with a matrix of molecular features, including retention time (RT), and mass/change ratio (*m/z*), was processed using the XCMS program. The identification of compounds is based on multiple dimensions such as RT, precise mass number, secondary fragments, and isotopic distribution. KEGG, Lipidmaps (v2.3), METLIN database, and LuMet Plant3.0 local database were used for identification and analysis. The intensity of peak data was further processed by an in-house software MetaX. For quality control, the identifications of precursor ions of the expected positive ion adduct with less than a 5 ppm error were defined using high-resolution MS.

Results

Sample sequence characteristics

In total, 585,049 raw tags were obtained in *T. cuspidata*, 554,417 in *T. mairei*, and 559,155 in *T. media*. The quality of sequencing data was evaluated by various parameters, including sequence numbers, sequence lengths, effective ratios, Q20/Q30 quality values, and GC content in each sample (**Table S2**). The number of valid reads per sample ranged from 7,822 to 62,334, with the average read length for each sample ranging mainly from 200 to 400 bp (Fig. 1a). A total of 271 OTUs were detected in all libraries, including 230 OTUs in *T. media*, 115 in *T. mairei*, and 177 in *T. cuspidata*. In detail, 52 OTUs were common among the three *Taxus* species, while 1 OTUs, 27 OTUs and 44 OTUs were unique in *T. cuspidata*, *T. mairei*, and *T. media*, respectively (Fig. 1b and **Table S3**). The abundance of OTUs in the three *Taxus* species was shown by a heatmap, suggesting significant differences in fungal communities among the three *Taxus* species (Fig. 1c).

Alpha and beta diversity analysis

The endophytic fungal communities in *Taxus* trees were significantly influenced by host plants. Analysis of the Chao1 dilution curve showed that the curve reached an asymptote, indicating that the sequencing depth was adequate (Fig. 2a). Based on the Chao1 and Observed_species indexes, the richness of endophytic fungal species in *T. mairei* is the highest, followed by *T. cuspidate* and *T. media* (Fig. 2b). Similarly, the analysis of Shannon index and Simpson parameters showed that *T. mairei* exhibited the highest diversity of endophytic fungi among the three *Taxus* species, followed by *T. media* and *T. cuspidata* (**Fig. S1a** and **b**). The UniFrac distance measurement and multivariate analysis were applied to calculate the differences in endophytic fungal communities among 45 *Taxus* samples. The UPGMA tree analysis showed that the endophytic fungal communities of *T. cuspidata* and *T. media* were clustered together, indicating a closer genetic relationship between *T. media* and *T. cuspidata* (Fig. 2c). PCoA revealed significant variations in fungal community composition among different *Taxus* trees (Fig. 2d). The highest variations in the microbiota of the sample groups were 40.31% (PCo1) and 28.66% (PCo2), highlighting the distinctions in endophytic fungi among the three *Taxus* species.

Taxonomic analysis of endophytic fungal communities

After filtering out the rare OTUs, the remaining OTUs were grouped into 2 phyla, 6 classes, and 19 genera (**Table S4**). The two identified phyla were Ascomycota and Basidiomycota, and the relative abundance of these phyla varied among the three *Taxus* species. No Basidiomycota fungi were detected in *T. cuspidate*. Ascomycota fungi accounted for 95.5% in *T. media* and only 99.0% in *T. mairei* (Fig. 3a).

Among the eight classes identified, the dominant class was Dothideomycetes, which accounted for 93.1%, 76.9% and 70.0% in *T. cuspidate*, *T. mairei*, and *T. media*, respectively. The second dominant class was Eurotiomycetes, accounting for 11.7% in *T. mairei* and 10.8% in *T. media*. However, no Eurotiomycetes fungi were detected in *T. cuspidate*. Ascomycota, an unidentified fungus in the phylum *Ascomycota*, was the second dominant class in *T. media*, accounting for 11.6%, and in *T. cuspidate*, accounting for 6.9%. No Ascomycota was detected in *T. mairei*. The remaining enriched classes were Sordariomycetes, Microbotryomycetes, Basidiomycota, and Lecanoromycetes. All the above fungal classes were not detected in *T. cuspidate*, while Basidiomycota and Lecanoromycetes were not detected in *T. mairei* (Fig. 3b). At the genus level, the dominant genus in *T. cuspidate* was *Lophiostoma* (75.6%), the dominant genera in *T. media* were *Capnodiales* (24.1%) and *Davidiella* (15.8%), and the dominant genera in *T. mairei* were *Ramichloridium*

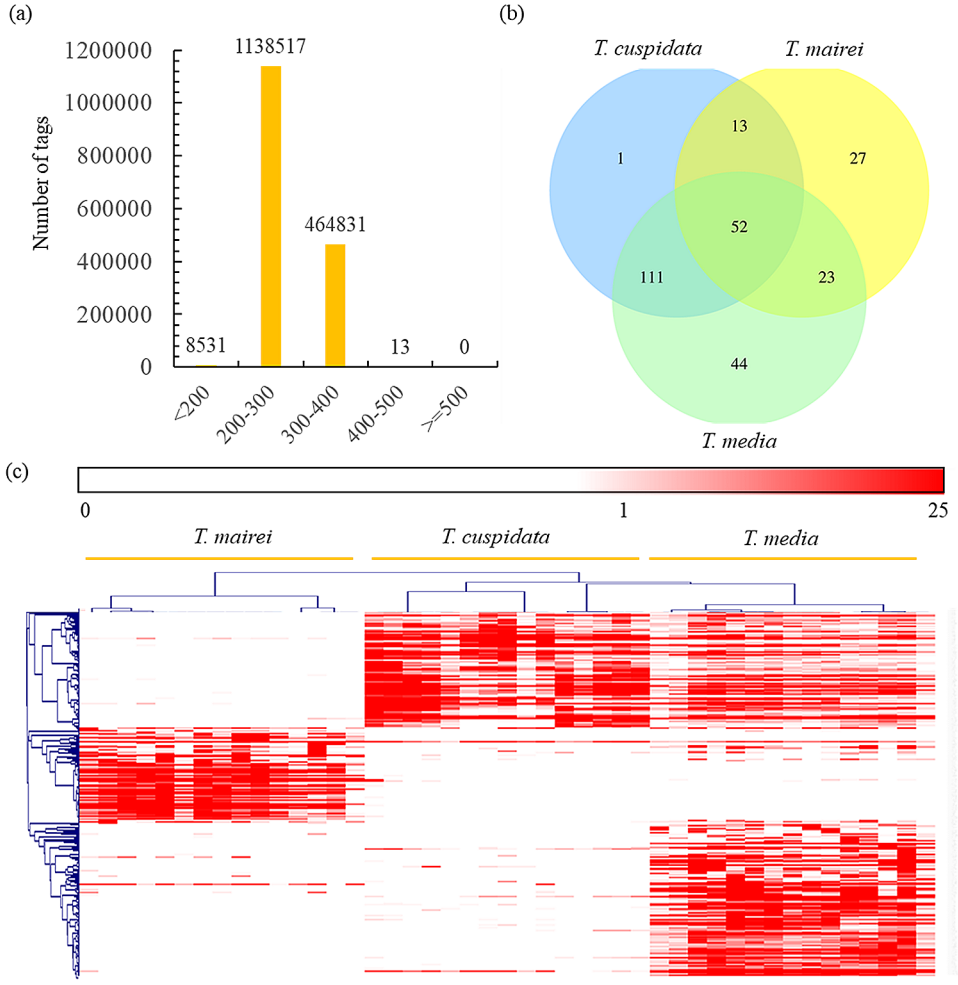

Fig. 1 Overview of the sequencing data. **(a)** The length of all tags. **(b)** Venn diagram shows the OTUs shared by *T. cuspidate*, *T. mairei*, and *T. media* samples. **(c)** A heatmap of the abundance of OTU in the three Taxus species (*N* = 15). The heatmap scale ranges from 0 to 25 on a log2 scale

(28.6%) and *Alternaria* (19.9%) (Fig. 3c). The detailed information of endophytic fungal communities is listed in **Table S4**.

Relationships between different fungal genera and the accumulation of taxoids

To reveal the relationship between the differential fungal genus and taxoid accumulation, a previously published untargeted metabolome was used for Pearson correlation analysis [32]. The detailed information regarding the relationship between the differential fungal genus and the accumulation of taxoids is presented in **Table S5**. *Ramichloridium*, *Alternaria*, *Guignardia*, *Glomerella*, and *Phyllosticta* were negatively correlated with the accumulation of Taxol (paclitaxel) (Rho < -0.99) (Fig. 4a). *Capnodiales*, *Devriesia*, *Ascomycota*, *Paramycosphaerella*, *Basidiomycota*, *Ceramothyrium*, and *Sporobolomyces* were positively correlated with the accumulation of 10-deacetyl-2-debenzoylbaccatin III (Rho < -0.99) (Fig. 4b). Most of the differential fungal genus were positively correlated with the accumulation of 3'-*N*-debenzoyl-2'-deoxytaxel and 3'-*N*-debenzoyltaxol (Fig. 4c and **d**). Most of the differential fungal genus, except for *Lophiostoma*, were negatively correlated with the accumulation of baccatin III and 10-deacetylbaccatin III (Fig. 4e and **f**).

Screening of taxol biosynthesis-related fungal isolates

ITS sequencing identified a *T. mairei*-specifically accumulated endophytic fungal genus, *Alternaria* (Fig. 5a). According to the Pearson correlation analysis, the *Alternaria* genus showed a negative correlation with the accumulation of Taxol (Rho = -0.93), suggesting its role in the negative regulation of the Taxol biosynthesis pathway (Fig. 5b). Twigs of *T. mairei* were used for screening the endophytic fungal isolates, and two *Alternaria* isolates, L7 and M14, were isolated from the twigs of *T. mairei* (Fig. 5c). The phylogenetic tree displayed similar taxonomic affinities among identifiable taxa and the two *Alternaria* isolates, L7 and M14 (Fig. 5d).

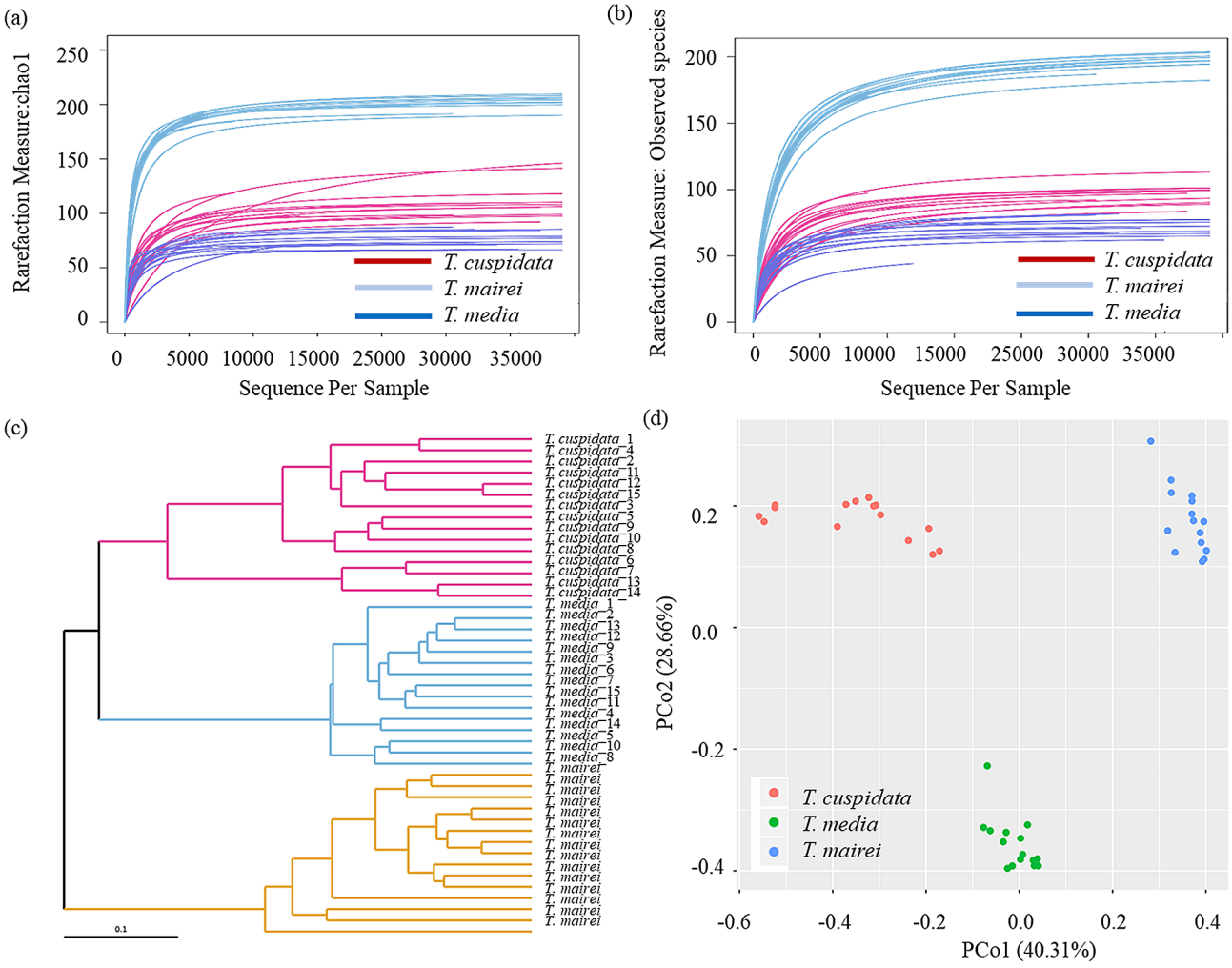

Fig. 2 Alpha diversity analysis. Four parameters, including **(a)** Rarefaction: Chao1 index curve, **(b)** Rarefaction: Observed_species index curve, **(c)** Dendrogram analysis and **(d)** PCA, were analyzed

Effect of*Alternaria* isolate inoculation on the expression pattern of *T. mairei*

To investigate the role of the *Alternaria* genus in Taxol biosynthesis, transcriptomic analysis was performed. Transcriptome study revealed that 4298 DEGs, including 1832 up- and 2466 down-regulated genes, were detected in the L7/CK comparison, and 4784 DEGs, including 2082 up- and 2702 down-regulated genes, were detected in the M14/Ck comparison (Fig. 6a). A Venn diagram illustrated that 3348 DEGs were common to both comparisons (Fig. 6b). GO enrichment analysis revealed that a number of DEGs were enriched in Taxol biosynthesis pathway-related GO terms, such as the paclitaxel biosynthetic process (GO:0042617, P=2.03E-11) and paclitaxel metabolic process (GO:004261, P=5.86E-23). Additionally, L7 and M14 inoculations activated several hormone biosynthesis and signaling transduction pathways (Fig. 6c).

A number of Taxol biosynthesis-related genes were analyzed by transcriptomic analysis. Interestingly, the expression levels of the most identified genes in the Taxol biosynthesis pathway were significantly inhibited by L7 and M14 inoculations (Fig. 6d). Most of the ethylene signaling pathway-related genes were significantly up-regulated by L7 and M14 inoculations. Meanwhile, most of the ABA signaling pathway-related genes were significantly down-regulated by L7 and M14 inoculations (Fig. 6e). Our data confirmed that *Alternaria* has a negative regulatory effect on the host's Taxol biosynthesis by regulating the host's hormone pathways.

Effect of *Alternaria* isolate inoculation on the metabolite accumulation of *T. mairei*

After 48 h incubation, the twigs of *T. mairei* were collected and cleaned for untargeted metabolomic analysis. In total, 7029 annotated metabolites were detected (**Table S6**). Quality control of the ion feature data was

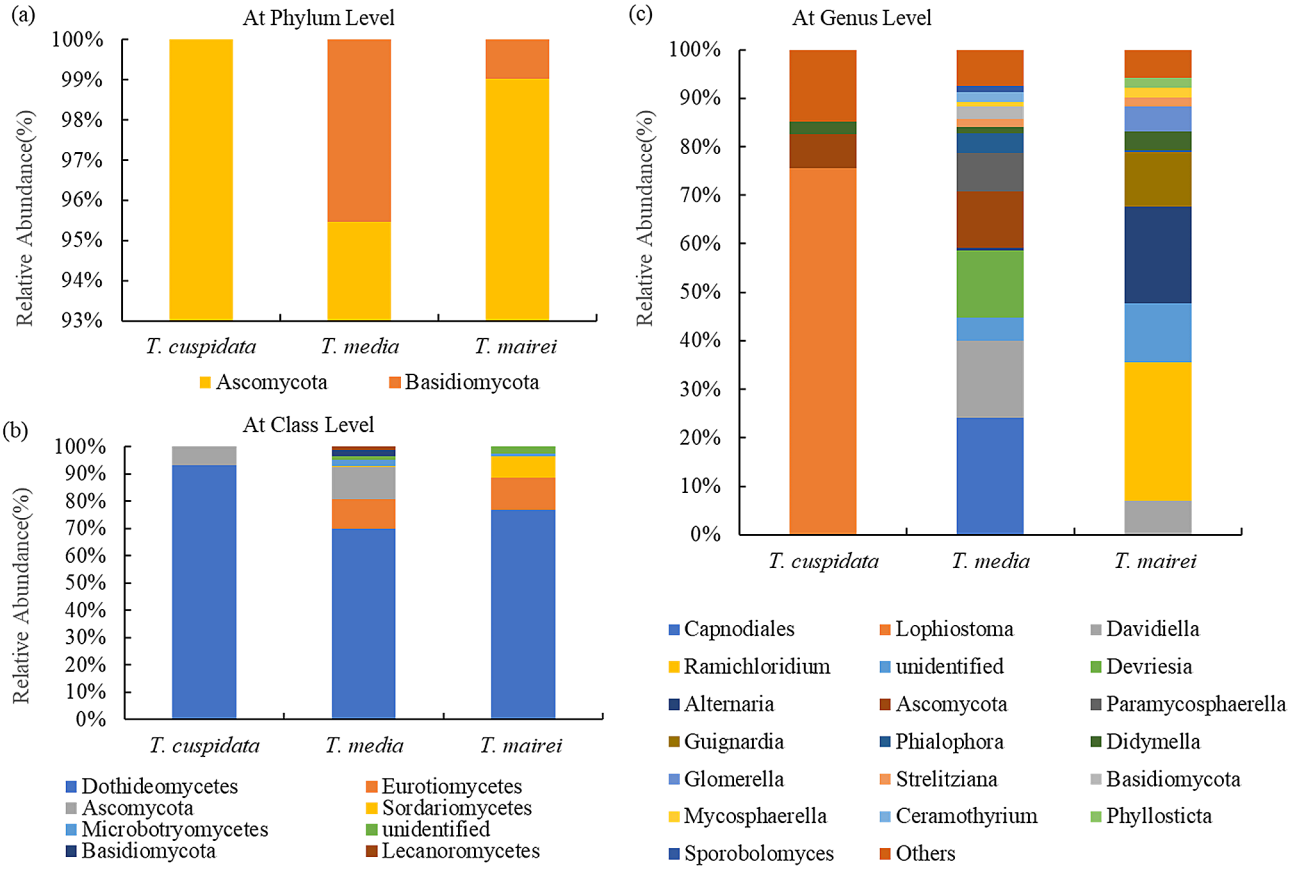

Fig. 3 Taxonomic analysis of endophytic fungal communities. **(a)** Taxonomic analysis of endophytic fungi at phylum level. **(b)** Taxonomic analysis of endophytic fungi at class level. **(c)** Taxonomic analysis of endophytic fungi at genus level

analyzed (**Fig. S1**), indicating good data repeatability. KEGG classification analysis identified four taxoids, including 10-deacetylbaccatin III, baccatin III, 10-deacetylytaxol, and taxol, and seven phytohormones, including (±) abscisic acid, (S)-(+)-abscisic acid, (+)-7-iso-jasmonate, (+)-7-iso-jasmonate, 24-epi-brassinolide, gibberellin A36, and gibberellin A37 (**Table S6**). Interestingly, the four detected taxoids in *T. mairei* were significantly inhibited by L7 inoculation. (±) Abscisic acid and (+)-7-iso-jasmonate were significantly up-regulated by the L7 inoculation, and gibberellin A36 and gibberellin A37 were significantly down-regulated by the L7 inoculation (Fig. 7).

Discussion

Recently, the diversity of plant endophyte communities has gradually become a hot topic. Endophytic fungi colonizing plant tissues have beneficial effects on the host ecosystem [35]. Endophytic fungi form a close relationship with host plants during long-term co-evolution, providing nutrients for plant defense and enhancing plant resistance [36]. Furthermore, endophytic fungi influence the growth and production of key secondary metabolites in host plants [37]. Our study analyzed the diversity of endophytic fungi in three different *Taxus* species, providing a foundation for studying the impact of plant-endophytic microbial interaction on the host's Taxol biosynthesis.

Flores-Cotera et al. screened the endophytic fungi of *T. globosa* and obtained 245 endophytes from 21 genera [22]. In 2013, 81 fungal isolates belonging to 8 genera were recovered in *T. media* based on morphological and molecular identification [23]. A total of 145 endophytic fungi, comprising 124 taxa, were isolated from the barks, branches, and leaves of *T. mairei* [38]. In the present study, the characteristics of endophytic fungi in *Taxus* were analyzed and compared. A total of 271 endophytic fungal OTUs were detected in three *Taxus* species, representing 19 genera, suggesting that our data provides sufficient coverage.

Altitude has a significant impact on the interaction between endophytic fungi and their hosts. Below an altitude of 2000 m, Ascomycota occupies a larger proportion of endophytic fungi than Basidiomycota, becoming the dominant phylum [39]. The distribution of endophytic fungi varied greatly among three *Taxus* species, primarily due to host genotype and growing environment [39]. In *T. cuspidate* and *T. mairei*, Ascomycota is the dominant

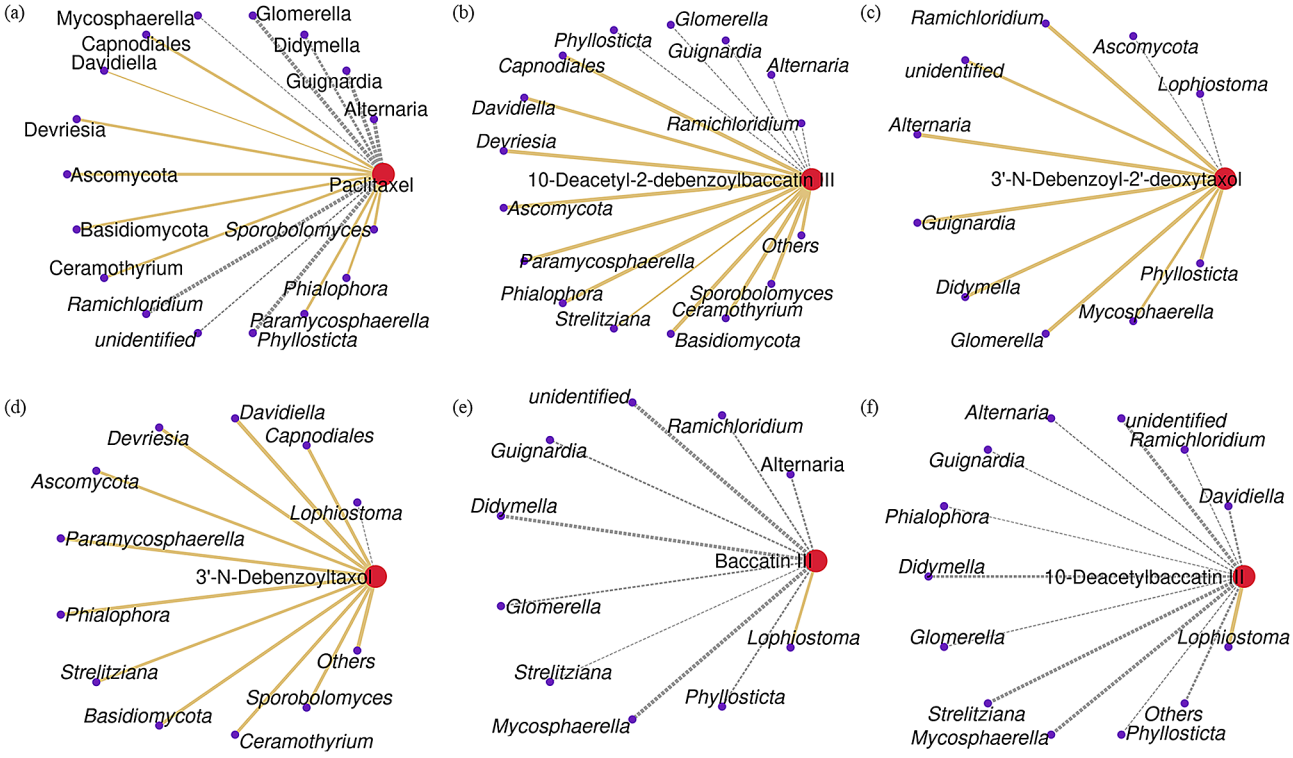

Fig. 4 Pearson correlation analysis of differential fungal genus and host's taxoid accumulation levels. Analysis of paclitaxel **(a)**, 3′-*N*-debenzoyltaxol **(b)**, 3′-*N*-debenzoyl-2′-deoxytaxol **(c)**, 10-deacetyl-2-debenzoylbaccatin III **(d)**, baccatin III **(e)**, and 10-deacetylbaccatin III **(f)**-related differential fungal genus

phylum, suggesting that these species are more sensitive to altitude than the hybrid variety *T. media*. In *T. globosa*, the Mexican yew, Dothideomycetes was considered one of the dominant classes [22]. In our study, Dothideomycetes and Eurotiomycetes were the predominant classes found in all *Taxus* trees.

At the genus level, *Alternaria* and *Davidiella* fungi are adapted to extreme low-temperature environments, exhibiting good adaptability to continuous low temperatures and high ultraviolet radiation [40]. Previous studies have also found evidence of altitude-related competition between *Alternaria* and *Davidiella* [25]. *Taxus* trees grow in areas with high altitudes and low temperatures [41]. Therefore, *Alternaria* and *Davidiella* play an important role in the adaptability of *Taxus* trees to low temperatures and high altitudes. In addition to environmental adaptation, endophytic fungi contain many important plant-derived secondary metabolites [42, 43]. Newly identified 3,4-dihydroisocoumarin derivatives were detected in the endophytic fungus *Lophiostoma* sp. Sigrf10, which is isolated from *Siraitia grosvenorii* [44]. Several novel compounds, including scorpinone, 5-deoxybostrycoidin, and 4-methyl-5,6-dihydro-2 H-pyran-2-one, were extracted from the endophytic fungus *Lophiostoma* sp. Eef-7, which is isolated from *Eucalyptus exserta* [26]. The *Lophiostoma* genus occupied the largest proportion in *T. cuspidata*, suggesting an essential role of *Lophiostoma* in *Taxus* secondary metabolism.

To date, a large number of Taxol-producing fungi have been identified, offering a potential shortcut for the industrial production of Taxol [45]. For example, *Alternaria alternata* F3 is the first reported taxol-producing endophytic fungus isolated from *Taxus* fruits [46]. Endophytic fungi have a significant impact on the growth, development, and taxane accumulation of *Taxus* trees. Three promising strains, *Kocuria* TRI2-1, *Micromonospora* TSI4-1, and *Sphingomonas* MG-2, were also found to significantly enhance the accumulation of taxanes in *T. yunnanensis* stem cells [47]. The endophytic fungus *Pseudodidymocyrtis lobariellae* KL27-FB has been found to significantly enhance the expression of key genes involved in the Taxol biosynthesis pathway [28]. Our study suggests that two *Alternaria* isolates (L7 and M14) may have a negative regulatory effect on Taxol biosynthesis by down-regulating the expression of Taxol biosynthesis-related genes. In *Taxus* tissues, taxoids play a role in the growth inhibition of certain specific endophytic fungi. Various endophytic fungi played opposite roles in maintaining the balance of Taxol biosynthesis in *Taxus* trees. In our study, two fungi negatively correlated with Taxol biosynthesis were screened, which can be used as antagonistic fungi to screen endophytic fungi positively correlated with Taxol biosynthesis.

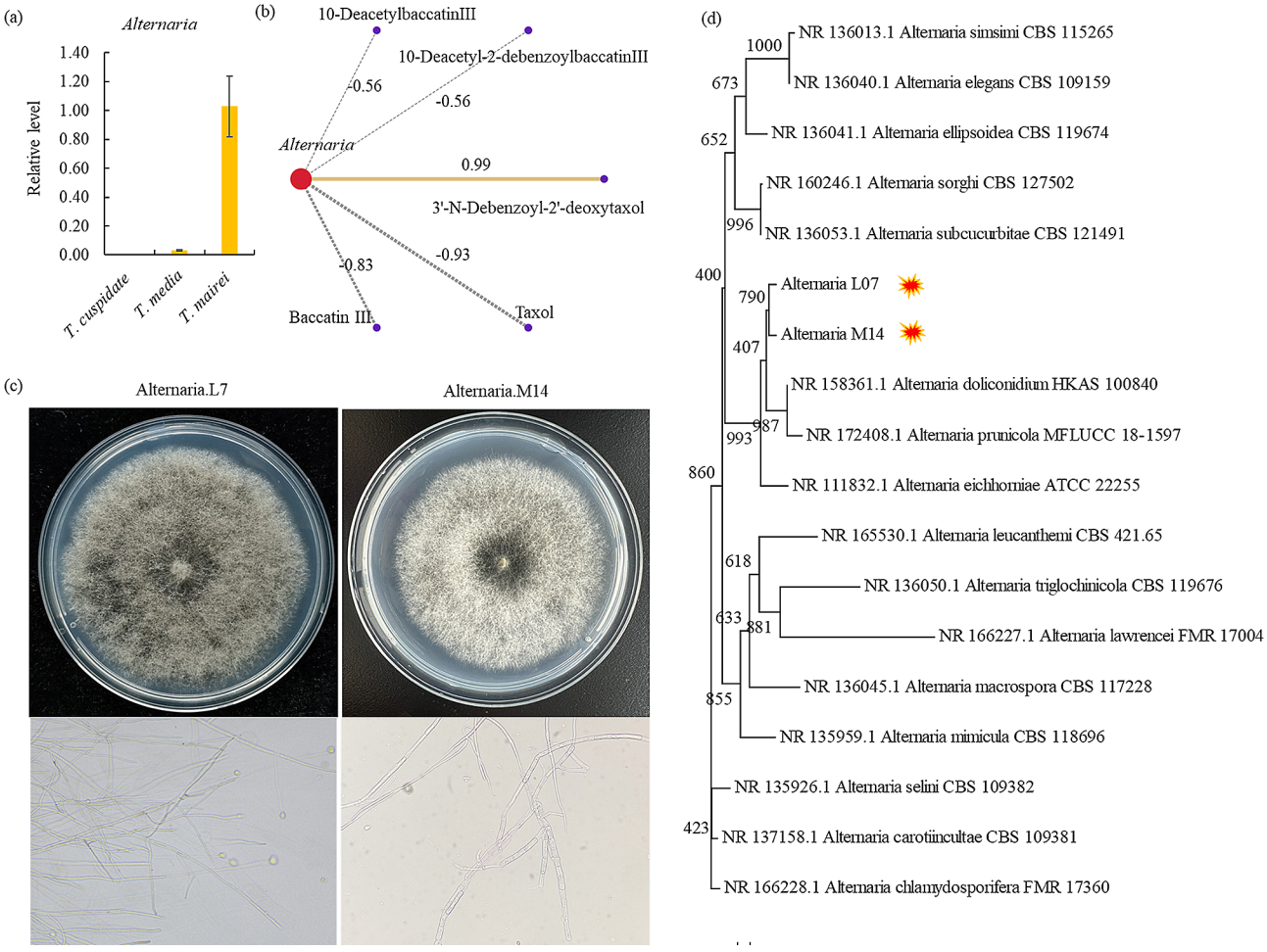

Fig. 5 Screening of taxol biosynthesis-related fungal isolates. **(a)** The relative abundance level of *Alternaria* genus in three different Taxus species. **(b)** Pearson correlation analysis of differential fungal genus and host's taxoid accumulation levels. **(c)** Photograph of two fungal isolates belonging to *Alternaria* genus. **(d)** Phylogenetic relationships of *Alternaria* isolates L7 and M14 with other Alternaria spp. based on the ITS sequences. Red stars indicated *Alternaria* isolates L07 and M14

Phytohormones, such as jasmonic acid (JA), gibberellic acid (GA), abscisic acid (ABA), and ethylene, have been reported to be involved in the regulation of Taxol biosynthesis [48]. Our RNA-seq analysis showed that most of the ethylene signaling pathway-related genes were significantly up-regulated under L7 and M14 infections, suggesting that *Alternaria* isolates might affect Taxol biosynthesis by activating the ethylene signaling pathway. Moreover, the expression of ABA signaling pathway genes was significantly down-regulated by L7 and M14 infections, indicating that ABA has the opposite effect of ethylene in the regulation of Taxol biosynthesis. Several hormones, such as JA, ABA, GA, and ET, were reported to be involved in Taxol biosynthesis [49, 50]. The two *Alternaria* strains might inhibit Taxol biosynthesis by regulating various hormone signaling pathways.

In summary, our study initially examined the variances in abundance and community of endophytic fungi among three *Taxus* species using high-throughput sequencing. A total of 271 OTUs representing 19 genera of fungi were detected in three library groups. Taxonomic analysis of endophytic fungal communities suggests significant differences in endophytic fungi among different *Taxus* trees. A Pearson correlation analysis was conducted to predict the presence of taxol biosynthesis-related fungal genera and to screen two *Alternaria* isolates. The two *Alternaria* isolates (L7 and M14) may have a negative regulatory effect on taxol biosynthesis by modulating hormone signaling pathways. Our findings will serve as a foundation for advancing research in the utilization of *Taxus* and will assist in the identification of endophytic fungi associated with taxol production.

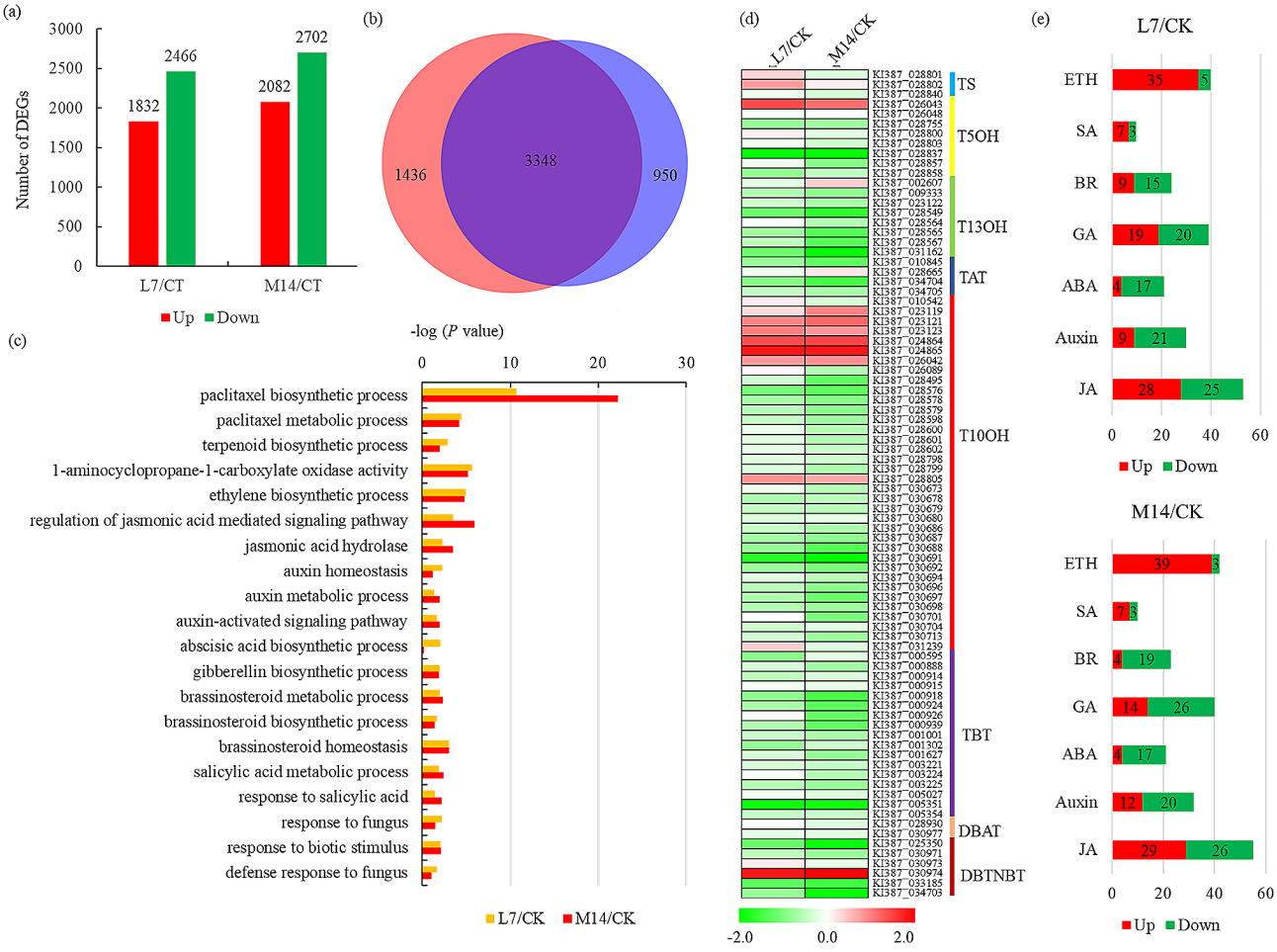

Fig. 6 Effect of inoculation with*Alternaria*isolates L7 and M14 on the host *Taxus* trees. **(a)** The spores of *Alternaria* isolate L7 and M14 was sprayed onto the twigs of T. mairei. **(b)** The number of up-regulated and down-regulated genes after *Alternaria* isolate L7 and M14 spore infection. **(c)** GO enrichment analysis of the DEGs after *Alternaria* isolate L7 and M14 spore infection. **(d)** The expression level of taxol biosynthesis pathway genes after *Alternaria* isolate L7 and M14 spore infection. Red indicated up-regulated genes and green indicated down-regulated genes. The heatmap scale ranges from − 2 to + 2 on a log2 scale. **(e)** The number of phytohormone-related DEGs. Red indicated up-regulated genes and green indicated down-regulated genes

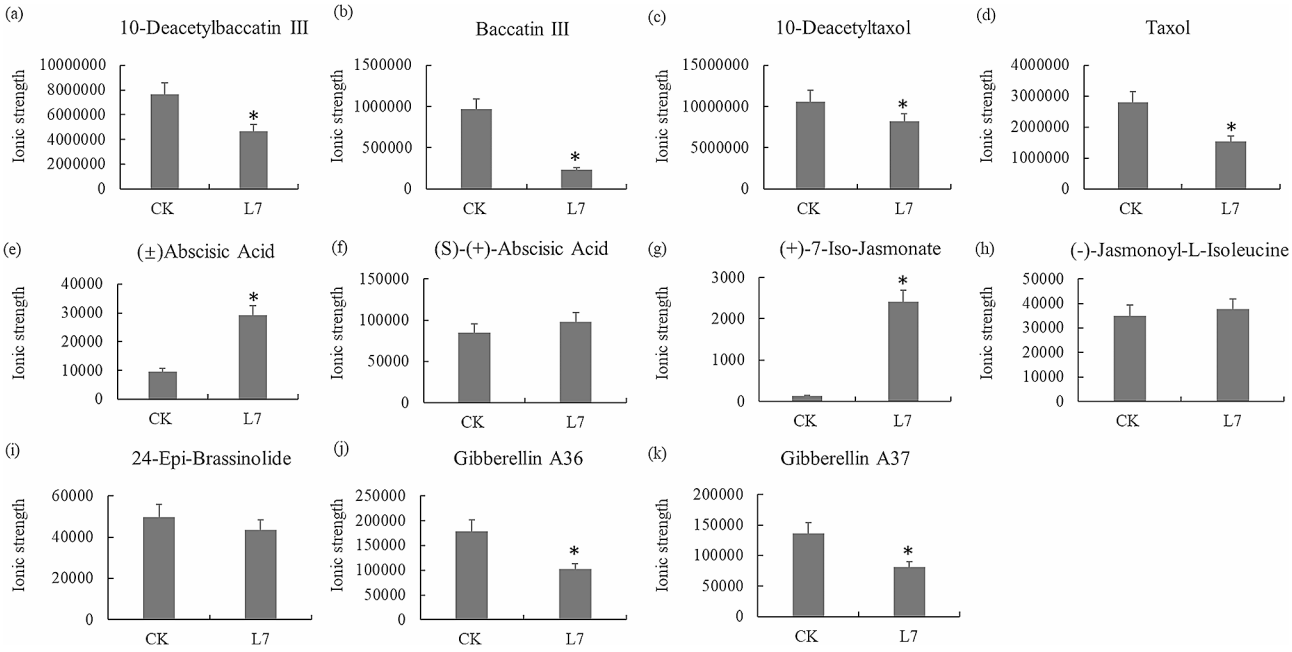

Fig. 7 Effect of inoculation with *Alternaria* isolate on the metabolite accumulation of *T. mairei*. Relative accumulation levels of four taxoids and seven hormones. Each value is the mean ± standard deviation of three biological repeats. *$P < 0.05$

Abbreviations

ITS	Internal Transcribed Spacer
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
FPKM	Fragment Per Kilobase of transcript per Million mapped reads
DEG	differentially expressed gene
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Gene Ontology
PDA	Potato Dextrose Agar
JA	jasmonic acid
GA	gibberellic acid
ABA	abscisic acid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03445-8.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

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Author contributions

Y.S. and C.S. conceptualized the initial study; H.Z., W.L., R.M., Y.Z., K.H., and X.L. were involved in the experimental layout; H.Z., W.L., Y.Z. Z.X., X.X., W.Z., S.T., and X.L. performed the lab experiments, H.Z., Y.S. and C.S. drafted the initial article; all authors discussed the results, reviewed the article, and approved the final article.

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Data availability

The raw data of ITS-seq was submitted to NCBI database (BioProject ID PRJNA1062300). Transcriptome data are available at the NCBI database (BioProject ID PRJNA1073191).

Declarations

Ethics approval and consent to participate

This project uses plant materials and does not utilize transgenic technology. The authorities responsible for the Taxus resources are the Mount Changbai National Nature Reserve, who provided permission to collect the samples of *T. cuspidata* and the Mount Tianmu National Nature Reserve, who provided permission to collect the samples of *T. mairei*, for our scientific research. *T. media* is cultivated variety purchased from the Wuwangnong company (Chengdu, China), who provided permission to use the seedlings for our scientific research.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Adherence to national and international regulations

Not application.

Conflict of interest

The authors have no conflicts of interest to declare.

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References

1. Yu C, Huang J, Wu Q, Zhang C, Li XL, Xu X, Feng S, Zhan X, Chen Z, Wang H, et al. Role of female-predominant MYB39-bHLH13 complex in sexually dimorphic accumulation of taxol in *Taxus media*. Hortic Res. 2022;9:uhac062.
2. Zhan X, Zang Y, Ma R, Lin W, Li XL, Pei Y, Shen C, Jiang Y. Mass Spectrometry-imaging analysis of active ingredients in the leaves of *Taxus cuspidata*. ACS Omega. 2024;9(16):18634–42.
3. Shao F, Wilson IW, Qiu D. The research progress of Taxol in *Taxus*. Curr Pharm Biotechnol. 2021;22(3):360–6.
4. Yu C, Hou K, Zhang H, Liang X, Chen C, Wang Z, Wu Q, Chen G, He J, Bai E, et al. Integrated mass spectrometry imaging and single-cell transcriptome atlas strategies provide novel insights into taxoid biosynthesis and transport in *Taxus mairei* stems. Plant J. 2023;115(5):1243–60.
5. Qiao X, Zhang J, Wang Z, Xu Y, Zhou T, Mi X, Cao M, Ye W, Jin G, Hao Z, et al. Foundation species across a latitudinal gradient in China. Ecology. 2021;102(2):e03234.
6. Long T, Wu X, Wang Y, Chen J, Xu C, Li J, Li J, Zang R. The population status and threats of *Taxus cuspidata*, a plant species with extremely small populations in China. Global Ecol Conserv. 2021;26:e01495.
7. Zhan X, Qiu T, Zhang H, Kailin H, Liang X, Chen C, Wang Z, Wu Q, Wang X, Li XL et al. Mass spectrometry imaging and single-cell transcriptional profiling reveal the tissue-specific regulation of bioactive ingredient biosynthesis in *Taxus* leaves. Plant Commun 2023:100630.
8. Feng S, Kailin H, Zhang H, Chen C, Huang J, Wu Q, Zhang Z, Gao Y, Wu X, Wang H, et al. Investigation of the role of TmMYB16/123 and their targets (TmMTP1/11) in the tolerance of *Taxus media* to cadmium. Tree Physiol. 2023;43(6):1009–22.
9. Saloustros E, Mavroudis D, Georgoulias V. Paclitaxel and Docetaxel in the treatment of breast cancer. Expert Opin Pharmacother. 2008;9(15):2603–16.
10. Yang YH, Mao JW, Tan XL. Research progress on the source, production, and anti-cancer mechanisms of paclitaxel. Chin J Nat Med. 2020;18(12):890–7.
11. Malhadas C, Malheiro R, Pereira JA, de Pinho PG, Baptista P. Antimicrobial activity of endophytic fungi from olive tree leaves. World J Microbiol Biotechnol. 2017;33(3):46.
12. Zhan X, Chen Z, Chen R, Shen C. Environmental and genetic factors involved in plant protection-associated secondary metabolite biosynthesis pathways. Front Plant Sci. 2022;13:877304.
13. Tian Y, Amand S, Buisson D, Kunz C, Hachette F, Dupont J, Nay B, Prado S. The fungal leaf endophyte *paraconiothyrium* variabile specifically metabolizes the host-plant metabolome for its own benefit. Phytochemistry. 2014;108:95–101.
14. Santoyo G, Moreno-Hagelsieb G, Orozco-Mosqueda Mdel C, Glick BR. Plant growth-promoting bacterial endophytes. Microbiol Res. 2016;183:92–9.
15. Stierle A, Strobel G, Stierle D, Grothaus P, Bignami G. The search for a taxol-producing microorganism among the endophytic fungi of the Pacific yew, *Taxus brevifolia*. J Nat Prod. 1995;58(9):1315–24.
16. Kumar P, Singh B, Thakur V, Thakur A, Thakur N, Pandey D, Chand D. Hyper-production of taxol from *Aspergillus Fumigatus*, an endophytic fungus isolated from *Taxus* sp. of the Northern Himalayan region. Biotechnol Rep (Amst). 2019;24:e00395.
17. Kasaei A, Mobini-Dehkordi M, Mahjoubi F, Saffar B. Isolation of Taxol-producing endophytic fungi from Iranian yew through novel molecular approach and their effects on human breast cancer cell line. Curr Microbiol. 2017;74(6):702–9.
18. Somjaipeng S, Medina A, Kwasna H, Ordaz Ortiz J, Magan N. Isolation, identification, and ecology of growth and taxol production by an endophytic strain of paraconiothyrium variabile from English yew trees (*Taxus baccata*). Fungal Biol. 2015;119(11):1022–31.
19. Stadler M, Kolarik M. Taxol is NOT produced sustainably by endophytic fungi ! – a case study for the damage that scientific papermills can cause for the scientific communities. Fungal Biology Reviews. 2024;49:100367.
20. Sadeghi F, Samsampour D, Seyahooei MA, Bagheri A, Soltani J. Diversity and spatiotemporal distribution of fungal endophytes associated with *Citrus reticulata* cv. Siyahoo. Curr Microbiol. 2019;76(3):279–89.
21. Hao DC, Song SM, Mu J, Hu WL, Xiao PG. Unearthing microbial diversity of *Taxus* Rhizosphere via MiSeq high-throughput amplicon sequencing and isolate characterization. Sci Rep. 2016;6:22006.
22. Soca-Chafre G, Rivera-Orduna FN, Hidalgo-Lara ME, Hernandez-Rodriguez C, Marsch R, Flores-Cotera LB. Molecular phylogeny and paclitaxel screening of fungal endophytes from Taxus Globosa. Fungal Biol. 2011;115(2):143–56.
23. Xiong ZQ, Yang YY, Zhao N, Wang Y. Diversity of endophytic fungi and screening of fungal paclitaxel producer from Anglojap yew, *Taxus x media*. BMC Microbiol. 2013;13:71.
24. Gauchan DP, Kandel P, Tuladhar A, Acharya A, Kadel U, Baral A, Shahi AB, Garcia-Gil MR. Evaluation of antimicrobial, antioxidant and cytotoxic properties of bioactive compounds produced from endophytic fungi of Himalayan yew (*Taxus wallichiana*) in Nepal. *F1000Res* 2020, 9:379.
25. Liu K, Ding X, Deng B, Chen W. Isolation and characterization of endophytic taxol-producing fungi from *Taxus Chinensis*. J Ind Microbiol Biotechnol. 2009;36(9):1171–7.
26. Mao Z, Zhang W, Wu C, Feng H, Peng Y, Shahid H, Cui Z, Ding P, Shan T. Diversity and antibacterial activity of fungal endophytes from Eucalyptus exserta. BMC Microbiol. 2021;21(1):155.
27. Salehi M, Moieni A, Safaie N, Farhadi S. Whole fungal elicitors boost paclitaxel biosynthesis induction in Corylus avellana cell culture. PLoS ONE. 2020;15(7):e0236191.
28. Cao X, Xu L, Wang J, Dong M, Xu C, Kai G, Wan W, Jiang J. Endophytic fungus *pseudodidymocyrtis* lobariellae KL27 promotes taxol biosynthesis and accumulation in *Taxus Chinensis*. BMC Plant Biol. 2022;22(1):12.
29. Wang C, Wu J, Mei X. Enhancement of taxol production and excretion in *Taxus chinensis* cell culture by fungal elicitation and medium renewal. Appl Microbiol Biotechnol. 2001;55(4):404–10.
30. Stierle A, Strobel G, Stierle D. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science. 1993;260(5105):214–6.
31. Shen C, Xue J, Sun T, Guo H, Zhang L, Meng Y, Wang H. Succinyl-proteome profiling of a high taxol containing hybrid Taxus species (*Taxus x media*) revealed involvement of succinylation in multiple metabolic pathways. Sci Rep. 2016;6:21764.
32. Zhou T, Luo X, Zhang C, Xu X, Yu C, Jiang Z, Zhang L, Yuan H, Zheng B, Pi E, et al. Comparative metabolomic analysis reveals the variations in taxoids and flavonoids among three Taxus species. BMC Plant Biol. 2019;19(1):529.
33. Wang Y, Xu X, Liu T, Wang H, Yang Y, Chen X, Zhu S. Analysis of bacterial and fungal communities in continuous-cropping ramie (Boehmeria nivea L. Gaud) fields in different areas in China. Sci Rep. 2020;10(1):3264.
34. Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics. 2010;26(2):266–7.
35. Yan L, Zhu J, Zhao X, Shi J, Jiang C, Shao D. Beneficial effects of endophytic fungi colonization on plants. Appl Microbiol Biotechnol. 2019;103(8):3327–40.
36. Wang Z, Zhu Y, Li N, Liu H, Zheng H, Wang W, Liu Y. High-throughput sequencing-based analysis of the composition and diversity of endophytic bacterial community in seeds of saline-alkali tolerant rice. Microbiol Res. 2021;250:126794.
37. Wu LS, Dong WG, Si JP, Liu JJ, Zhu YQ. Endophytic fungi, host genotype, and their interaction influence the growth and production of key chemical components of Dendrobium catenatum. Fungal Biology. 2020;124(10):864–76.
38. Wu L, Han T, Li W, Jia M, Xue L, Rahman K, Qin L. Geographic and tissue influences on endophytic fungal communities of Taxus chinensis var. Mairei in China. Curr Microbiol. 2013;66(1):40–8.
39. Liu L, Ma M, Liu Z, Zhang L, Zhou J. Community structure of fungal pathogens causing spikelet rot disease of naked oat from different ecological regions of China. Sci Rep. 2021;11(1):1243.
40. Rafiq M, Nadeem S, Hassan N, Hayat M, Sajjad W, Zada S, Sajjad W, Hasan F. Fungal recovery and characterization from Hindu Kush mountain range, Tirich Mir glacier, and their potential for biotechnological applications. J Basic Microbiol. 2020;60(5):444–57.
41. Sreekanth D, Sushim GK, Syed A, Khan BM, Ahmad A. Molecular and morphological characterization of a taxol-producing endophytic fungus, *gliocladium* sp., from *Taxus baccata*. Mycobiology. 2011;39(3):151–7.
42. Deng Z, Li C, Luo D, Teng P, Guo Z, Tu X, Zou K, Gong D. A new cinnamic acid derivative from plant-derived endophytic fungus *pyronema* sp. Nat Prod Res. 2017;31(20):2413–9.
43. Fatima N, Kondratyuk TP, Park EJ, Marler LE, Jadoon M, Qazi MA, Mehboob Mirza H, Khan I, Atiq N, Chang LC, et al. Endophytic fungi associated with *Taxus fuana* (West Himalayan Yew) of Pakistan: potential bio-resources for cancer chemopreventive agents. Pharm Biol. 2016;54(11):2547–54.
44. Mao Z, Xue M, Gu G, Wang W, Li D, Lai D, Zhou L. Lophiostomin A-D: new 3,4-dihydroisocoumarin derivatives from the endophytic fungus *lophiostoma* sp. Sigrf10. RSC Adv. 2020;10(12):6985–91.

45. Zhou X, Zhu H, Liu L, Lin J, Tang K. A review: recent advances and future prospects of taxol-producing endophytic fungi. Appl Microbiol Biotechnol. 2010;86(6):1707–17.
46. Fu Y, Li X, Yuan X, Zhang Z, Wei W, Xu C, Song J, Gu C. *Alternaria alternata* F3, a novel taxol-producing endophytic fungus isolated from the fruits of *Taxus cuspidata*: isolation, characterization, taxol yield improvement, and antitumor activity. Appl Biochem Biotechnol. 2023;196(4):2246–69.
47. Liu Q, Li L, Chen Y, Wang S, Xue L, Meng W, Jiang J, Cao X. Diversity of endophytic microbes in *Taxus yunnanensis* and their potential for plant growth promotion and taxane accumulation. Microorganisms. 2023;11(7):1645.
48. Zheng H, Fu X, Shao J, Tang Y, Yu M, Li L, Huang L, Tang K. Transcriptional regulatory network of high-value active ingredients in medicinal plants. Trends Plant Sci. 2023;28(4):429–46.
49. Demidova E, Globa E, Klushin A, Kochkin D, Nosov A. Effect of methyl jasmonate on the growth and biosynthesis of C13- and C14-hydroxylated taxoids in the cell culture of yew (*Taxus Wallichiana* Zucc.) Of different ages. Biomolecules. 2023;13(6):969.
50. Cao X, Xu L, Li L, Wan W, Jiang J. TcMYB29a, an ABA-responsive R2R3-MYB transcriptional factor, upregulates Taxol biosynthesis in *Taxus Chinensis*. Front Plant Sci. 2022;13:804593.

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