

## Postprint: Isolation and Identification of Root Symbiotic Fungi in Young *Quercus wutaishanica* from the Liupan Mountains

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### Abstract

This study isolated culturable symbiotic fungi from the root systems of young *Quercus wutaishanica* of different ages, identified their species through a combination of colony morphology and molecular biology methods, and elucidated the community structure and dynamic changes of culturable symbiotic fungi in the roots of young *Quercus wutaishanica*. The results demonstrated: (1) A total of 249 fungal strains were isolated from the roots of young *Quercus wutaishanica*, which were identified as belonging to 2 phyla, 5 classes, 7 orders, 8 families, 15 genera, and 18 species, among which 2 species belonged to Basidiomycetes and 16 species belonged to Ascomycota. At the species level, *Pezizula pruinosa* exhibited the highest isolation frequency, accounting for 81.93% of the total isolated strains; *Dactylonectria torresensis*, *Ilyonectria robusta*, and *Atrocalyx nordicus* followed, accounting for 4.02%, 2.01%, and 2.01% of the total isolated strains, respectively. (2) The species and quantity of symbiotic fungi displayed substantial variations among *Quercus wutaishanica* of different tree ages, with the highest isolation frequency observed in 4-5-year-old plants (44.98%), followed by three-year-old plants (29.32%), two-year-old plants (19.68%), and one-year-old plants (6.02%). In conclusion, the roots of young *Quercus wutaishanica* in the Liupan Mountains of Ningxia harbor a rich diversity of culturable symbiotic fungi, with both species richness and abundance increasing with tree age; *Pezizula pruinosa* represents the absolutely dominant species among culturable symbiotic fungi in the roots of young *Quercus wutaishanica* in the Liupan Mountains. These findings establish a foundation for further exploitation of plant symbiotic microbial resources and investigation into the adaptation mechanisms underlying interactions between *Quercus wutaishanica* and symbiotic microorganisms under local adverse environmental conditions.

## Full Text

# Isolation and Identification of Symbiotic Fungi in Roots of Young *Quercus wutaishansea* in the Liupan Mountain Region

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## Abstract

This study isolated culturable symbiotic fungi from the roots of young *Quercus wutaishansea* trees of different ages and identified the species using a combination of colony morphology and molecular biology methods to elucidate the community structure and dynamic changes of culturable symbiotic fungi in young *Q. wutaishansea* roots. The results showed that: (1) A total of 249 fungal strains were isolated from young *Q. wutaishansea* roots, which were identified as 18 species belonging to 15 genera, 8 families, 7 orders, 5 classes, and 2 phyla. Among them, 2 species belonged to Basidiomycota and 16 species belonged to Ascomycota. At the species level, *Pezicula pruinosa* exhibited the highest isolation frequency, accounting for 81.93% of the total isolated strains, followed by *Dactylonectria torresensis* (4.02%), *Ilyonectria robusta* (2.01%), and *Atrocalyx nordicus* (2.01%). (2) The species composition and abundance of symbiotic fungi showed substantial variation across different tree ages, with the highest isolation frequency observed in 4-5-year-old plants (44.98%), followed by triennial plants (29.32%), biennial plants (19.68%), and annual plants (6.02%). In conclusion, the roots of young *Q. wutaishansea* in the Liupan Mountains harbor a rich diversity of culturable symbiotic fungi, with both species richness and abundance increasing with tree age. *Pezicula pruinosa* represents the absolutely dominant species among culturable symbiotic fungi in young *Q. wutaishansea* roots in this region. These findings establish a foundation for further exploration of plant symbiotic microbial resources and investigation of the adaptive mechanisms underlying the interaction between *Q. wutaishansea* and its symbiotic microorganisms in local stressful environments.

**Keywords:** Liupan Mountain; *Quercus wutaishansea*; symbiotic fungi; isolation and culture; identification

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*Quercus wutaishansea* is widely distributed across northern China, characterized by its tolerance to cold, drought, and barren conditions. It often forms patchy forests or single-dominant communities near mountain tops and ridges, serving as a primary constructive species in northern forest vegetation (Zhang et al., 2021; Lian et al., 2022). The Liupan Mountains are located in the central-

western part of the Loess Plateau, within the ecologically fragile transition zone between the northern agro-pastoral ecotone and the warm temperate semi-humid to semi-arid region. With a forest coverage rate of 70%, this area is known as the “wet island” of the Loess Plateau and a natural barrier for the Guanzhong Plain. The *Q. wutaishansea* secondary forest represents the dominant and climax community in the Liupan Mountain region, holding significant research value for maintaining local ecological balance (Wang, 2009).

In nature, plant organs host numerous mutualistic symbiotic fungi, with the greatest diversity found in roots, primarily including ectomycorrhizal fungi (ECMF), arbuscular mycorrhizal fungi (AMF), and dark septate endophytes (DSE) (Liu & Wang, 2018). Symbiotic fungi play crucial ecological roles in maintaining healthy plant ecosystems by enhancing water and mineral nutrient absorption, promoting plant growth, and improving stress resistance, particularly during plant recovery and ecological restoration under adverse conditions (Wang et al., 2018; Zobel et al., 2024). Mycorrhizal fungi connect roots to surrounding soil through extended hyphal networks, expanding root surface area to facilitate water and nutrient uptake and transport (Fors et al., 2023). DSE are abundant in stressful environments such as drought, nutrient-poor, and heavy metal-contaminated habitats. By regulating superoxide dismutase, soluble proteins, and melanin content, DSE enhance plant adaptation to drought and improve stress tolerance (González-Teuber et al., 2018; Liu & Wei, 2019). In cadmium-contaminated environments, DSE promote plant growth and limit metal toxicity to hosts by modifying root morphology and restricting heavy metal ion translocation (Chen et al., 2023).

Previous studies have demonstrated that *Q. wutaishansea* roots harbor abundant symbiotic fungi whose community structure changes dynamically with tree age. Using morphological characteristics of mycorrhizae and ITS sequences from root tips, Wang et al. (2017) identified 220 ectomycorrhizal fungal species from five typical *Q. wutaishansea* forests in northern China (Taibai Mountain, Ziwuling Mountain, Lingkong Mountain, Dongling Mountain, and Xiangshan), with richness positively correlated with altitude and soil organic matter. Wang et al. (2012) employed molecular methods to study ectomycorrhizal fungal communities in *Q. wutaishansea* of different ages, identifying 66 species—51 belonging to Basidiomycota and 15 to Ascomycota—with *Cenococcum geophilum* as the dominant fungus, and found that tree age significantly affected the ectomycorrhizal fungal community structure.

While existing molecular techniques have provided extensive data on symbiotic fungal diversity in *Q. wutaishansea*, isolation and purification of fungal mycelia are prerequisites for analyzing plant-symbiont interactions. The diversity characteristics of culturable symbiotic fungi in *Q. wutaishansea* roots remain unknown. Therefore, this study employed direct root segment isolation to separate and identify symbiotic fungi from young *Q. wutaishansea* roots in the Liupan Mountain region, aiming to clarify the community structure and dynamic patterns of culturable symbiotic fungi and provide fundamental data

and theoretical basis for future research on the interaction mechanisms between *Q. wutaishansea* and its symbiotic fungi.

### 1.1 Study Area Overview

The Liupan Mountain region covers a total area of 90,071 hm<sup>2</sup>, with elevations ranging from 2,200 to 2,900 m. The area has an average annual temperature of 5.9 °C, mean annual precipitation of 676 mm, and average annual relative humidity of 69%, characterized by a warm temperate semi-humid continental monsoon climate (Wang, 1988). *Q. wutaishansea* is primarily distributed on gentle slopes and shady, semi-shady, and semi-sunny slopes between 1,100 and 1,650 m elevation in the Liupan Mountain region.

### 1.2 Sample Collection

From July to September 2022, three 50 m × 50 m pure *Q. wutaishansea* forest plots were selected in the Qiuqianjia scenic area of the Liupan Mountain National Nature Reserve. Based on the age classification standards for *Q. wutaishansea* populations (Zhang et al., 2014) and the actual growth conditions in Liupan Mountain, young *Q. wutaishansea* trees of four different age classes (annual, biennial, triennial, and 4-5-year-old) were selected as study subjects. Following the five-point sampling method, five entire root systems were collected for each age class, totaling 60 samples. During sampling, surface cover around the plant base was removed with a shovel, and whole plants were carefully excavated. All roots were placed in ziplock bags with a small amount of surrounding soil for preservation. Collected root samples were refrigerated at 4 °C and transported to the laboratory within three days for subsequent processing.

### 1.3 Isolation of Root Symbiotic Fungi

In the laboratory, root samples were placed in petri dishes with water and soaked until surface soil softened. Fine brushes were used to gently remove soil from root surfaces and eliminate other plant roots. Roots were rinsed 3-5 times with distilled water. Direct root segment culture was used for isolating root symbiotic fungi. Potato dextrose agar (PDA) medium (potato 200 g · L<sup>-1</sup>, glucose 20 g · L<sup>-1</sup>, agar powder 16 g · L<sup>-1</sup>) was used for isolation and purification. After cooling to approximately 45 °C, ampicillin was added to a final concentration of 50 mg/L to inhibit bacterial growth. Thirty terminal 2-cm root segments were selected from each sample. Different sterilization methods were applied based on tree age: for annual and biennial roots, a two-step sterilization was performed by soaking in 70% ethanol for 2 min, then in 0.1% mercuric chloride for 40 s, followed by three rinses with sterile water each time, with final moisture absorption using sterile filter paper. For triennial and 4-5-year-old roots, an additional step was included: after the two-step sterilization, roots were soaked again in 70% ethanol for 1 min, rinsed three times with sterile water, and dried. Sterilized roots were cut into 0.2-0.3 cm segments, with four segments placed on each PDA plate. Each sample was inoculated with 100 root segments and incubated in a

biochemical incubator at 25 °C in darkness. Colony purification followed the method of Rousitamu et al. (2022), and colony morphological characteristics were described according to McLean et al. (1999) and Johansson et al. (2001), with detailed records of colony color, texture, shape, exudate presence, margin color and shape, and diameter. All isolated and purified strains were preserved in the Microbial Culture Collection of the Key Laboratory for Development and Utilization of Microbial Resources in Special Environments of Ningxia at North Minzu University.

## 1.4 Molecular Identification

**1.4.1 Fungal DNA Extraction** After purified mycelia covered the solid plates, a triangular scraper made of iron wire was used to collect surface mycelia under sterile conditions. Approximately 0.5 g of mycelia was weighed and DNA was extracted using the Sangon Biotech UNIQ-10 Column Fungal Genomic DNA Extraction Kit according to the manufacturer' s instructions.

**1.4.2 PCR Amplification of Fungal rDNA ITS Sequences** The universal fungal primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for colony PCR amplification. The 50  $\mu$ L reaction system contained: 2 $\times$ Hief PCR Master Mix 25  $\mu$ L, forward primer (10  $\mu$ mol  $\cdot$  L<sup>-1</sup>) 2.5  $\mu$ L, reverse primer (10  $\mu$ mol  $\cdot$  L<sup>-1</sup>) 2.5  $\mu$ L, template DNA 2  $\mu$ L, and ddH<sub>2</sub>O added to 50  $\mu$ L. The reaction conditions are shown in Table 1. Qualified samples were sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing.

**1.4.3 ITS Sequence Analysis** Fungal sequencing results were analyzed following the method of Li et al. (2023) to construct phylogenetic trees.

## 1.5 Statistical Analysis of Root Symbiotic Fungal Quantities

Isolation frequency analysis was used to determine the abundance of different symbiotic fungi in young *Q. wutaishansea* roots. Isolation frequency (%) = (Number of strains of a particular symbiotic fungus / Total number of isolated strains)  $\times$  100.

## 2.1 Morphological Characteristics of Root Symbiotic Fungal Colonies

A total of 249 fungal strains were isolated and purified from young *Q. wutaishansea* roots, which were classified into 18 morphological types based on colony characteristics (Figure 1 [Figure 1: see original paper], Table 2). Most colonies were circular, with a few being oval. Colors varied among white, yellow-white, gray, gray-black, pale-brown, and black. Most colonies did not produce pigments, and different colony types showed substantial variation in growth rate, with most growing slowly. Colonies T5, T12, T15, and T16 had thin waxy texture, while the rest were felted. Colony margins were white for T1, T4, T5, and

T11; milky white for T6; black for T18; and dark gray for T16. Exudates were observed as brown water droplets on T11, transparent water droplets on T12 and T15, light-yellow water droplets on T8, and black-brown water droplets on T3 and T18. Colonies T2, T14, and T16 grew fastest, reaching diameters of 7.3-7.5 cm, 7.2-7.5 cm, and 7.1-7.5 cm, respectively, after 30 days of purification. Colonies T1 and T10 grew slowest, reaching only 2.2-2.5 cm and 2.5-2.6 cm, respectively, after 30 days.

## 2.2 Molecular Identification Results

After splicing and quality control of rDNA ITS fragments from the 249 isolated strains, BLAST analysis against the GenBank database revealed similarity rates of 87.01%-100% with homologous sequences (Table 3). Based on colony morphotypes, 39 representative strains were selected from the 249 colonies. A phylogenetic tree was constructed using reference type strain sequences and homologous sequences (Figure 2 [Figure 2: see original paper]), revealing that the isolated strains belonged to 2 phyla, 5 classes, 7 orders, 8 families, 15 genera, and 18 species (Table 4). Representative strains L2 and L105 of morphotypes T1 and T2 clustered on branch V, belonging to the class Agaricomycetes of Basidiomycota. The remaining 37 representative strains all belonged to Ascomycota: six strains (L15, L54, L16, L100, L55, L21) clustered on branch I, belonging to Dothideomycetes; 12 strains (L81, L107, L120, L8, L65, L108, L66, L167, L159, L145, L184, L47) clustered on branch II, belonging to Leotiomycetes; two strains (L176, L151) clustered on branch III, belonging to Eurotiomycetes; and 17 strains (L128, L178, L69, L182, L133, L46, L126, L4, L43, etc.) clustered on branch IV, belonging to Sordariomycetes.

## 2.3 Isolation Frequency

Different fungal species showed substantial variation in isolation abundance and frequency (Table 3). *Pezizula pruinosa*, *Dactylonectria torresensis*, *Ilyonectria robusta*, and *Atrocalyx nordicus* exhibited the highest isolation frequencies at 81.93%, 4.02%, 2.01%, and 2.01%, respectively. The genus *Pezizula* was the dominant taxon with the highest isolation frequency (81.93%) across all developmental stages of *Q. wutaishansea*.

The abundance and isolation frequency of culturable symbiotic fungi varied significantly among different tree ages (Table 5). Fifteen strains were isolated from annual plants (6.02% isolation frequency), 49 strains from biennial plants (19.68%), 73 strains from triennial plants (29.32%), and 112 strains from 4-5-year-old plants (44.98%). The isolation frequency decreased in the order: 4-5-year-old > triennial > biennial > annual.

The results demonstrate that young *Q. wutaishansea* roots harbor a rich diversity of culturable symbiotic fungi, with Ascomycota as the dominant group. Previous molecular studies identified Basidiomycota (particularly ectomycorrhizal fungi) as the dominant symbiotic fungi in *Q. wutaishansea* roots. This study

provides an important methodological and empirical complement to existing research, completing the picture of symbiotic fungal diversity in *Q. wutaishansea* roots and laying the groundwork for investigating plant-symbiont interaction mechanisms and applications in seedling cultivation, afforestation, and ecosystem restoration.

Significant differences in the abundance and isolation frequency of symbiotic fungi were observed across different growth stages of *Q. wutaishansea*, with both species richness and abundance increasing with tree age. Community structure of symbiotic fungi shows dynamic changes during plant growth, though these patterns vary across studies. Rousitamu et al. (2022) found that symbiotic fungi isolated from *Picea crassifolia* roots varied substantially among tree ages, with highest isolation frequency in sapling stages, followed by seedling stages, and lowest in mature trees. Zheng et al. (2022) reported that 3-year-old *Phyllostachys edulis* had the highest endophytic fungal diversity, while 4-year-old plants had the lowest, indicating that bamboo age significantly alters endophytic fungal community composition. Séné et al. (2016) found that mature *Coccoloba uvifera* had significantly higher mycorrhizal fungal diversity and abundance than seedlings, whereas Corrales et al. (2016) observed no significant differences in ectomycorrhizal community structure between seedlings and mature trees of *Oreomunnea mexicana*. Symbiotic fungal community dynamics are constrained by multiple ecological factors including vegetation type, plant organ, and soil conditions, requiring comprehensive consideration of various factors to reveal underlying patterns.

This study marks the first isolation of *Pezicula* fungi from *Q. wutaishansea* roots, where they represent the absolutely dominant taxon. Zheng and Zhuang (2022) identified approximately one-third of 211 *Pezicula* strains from gymnosperms (e.g., pine, spruce, larch, and fir), with lower isolation frequency from angiosperms. The abundant colonization of *Pezicula* in *Q. wutaishansea* roots suggests a broader host range for this genus than previously known. Although *Pezicula* commonly colonizes various plant organs, isolation frequency is highest from roots (Yuan & Verkley, 2015; Ma et al., 2021).

*Pezicula* exhibits significant biocontrol and antifungal capabilities, producing various bioactive compounds such as echinocandins that protect plants from pathogenic fungi while possessing unique self-resistance mechanisms that render them unaffected by fungicides (Yue et al., 2018; Xu et al., 2019). *Pezicula* species produce multiple antibiotics and volatile organic compounds that reduce incidence and development of plant pathogenic fungi causing sclerotinia disease and northern corn leaf blight (Zhang et al., 2018). Antimicrobial experiments with *Pezicula* isolated from *Forsythia viridissima* demonstrated strong inhibition against *Botrytis cinerea* and *Verticillium* wilt (Wang et al., 2014). We hypothesize that as a major constructive species in northwestern China, the abundant *Pezicula* colonization in *Q. wutaishansea* roots may positively influence its growth and resistance to diseases and pests.

In summary, young *Q. wutaishansea* roots harbor a rich diversity of culturable

symbiotic fungi dominated by Ascomycota. Annual plants showed the lowest isolation frequency (6.02%), while 4-5-year-old plants showed the highest (44.98%), with species richness and abundance increasing with tree age. *Pezicula pruinosa* was the most frequently isolated dominant species (81.93%) in young *Q. wutaishansea* roots, representing the absolutely dominant taxon among culturable symbiotic fungi in the Liupan Mountain region. The interaction between this fungus and *Q. wutaishansea* warrants further investigation.

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