

The diversity of endophytic fungi in *Fagopyrum cymosum* and the beneficial strain JQ_R2 enhance the host's drought resistance through folate metabolism

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Abstract

Background

The emerging field of endophytic fungi studies their capability to enhance host plant growth and resistance through interactions. *F. cymosum*, a renowned medicinal plant, harbors a diverse yet inadequately understood array of endophytic fungi. Moreover, this plant, already at risk of endangerment, confronts additional threats posed by elevated temperatures and prolonged drought conditions. Hence, we aimed to identify drought-resistant endophytic fungi present in *F. cymosum* as a potential solution to alleviate this issue.

Results

The diversity of endophytic fungi across different habitats and tissue sections of *F. cymosum* was assessed using high-throughput sequencing technology in this investigation. Through correlation analysis, isolation, identification, and in vitro assays, we identified three target strains (JQ_R2, JQ_R14, JQ_L5) demonstrating growth-promotion and drought-resistance activities. These capabilities were subsequently confirmed in soil and hydroponic experiments involving *F. cymosum*. Transcriptomic and metabolomic analyses indicate that JQ_R2 enhances drought resistance in *F. cymosum* by boosting basal folate metabolism. Follow-up experiments validated the role of folate in aiding *F. cymosum*'s drought resistance and demonstrated that the JQ_R2 strain produces folate consistently under both normal and drought conditions. During drought conditions, the dihydrofolate reductase (DHFR) activity of the JQ_R2 strain was substantially increased, suggesting that the JQ_R2 strain enhances drought resistance in *F. cymosum*, potentially via modulation of the folate metabolism pathway.

Conclusions

In conclusion, our study illustrates that *F. cymosum* plants thriving in arid regions host a more diverse array of drought-resistant endophytic fungi, with the JQ_R2 strain augmenting drought resistance in *F. cymosum* by boosting basal folate metabolism. This finding sheds light on the operational mechanism of drought-resistant fungal strains, bearing considerable importance for forthcoming research on endophytic fungi and mitigating agricultural drought challenges.

Introduction

Fagopyrum cymosum (Trev.) Meisner is a perennial herbaceous plant member of the Polygonaceae family [1]. This species is predominantly found in China, India, Vietnam, and several other countries, with China serving as its primary distribution region [2]. The plant, known for its medicinal and edible qualities, yields seeds commonly used to produce flour-based foods. Its stems and leaves serve as fodder [3], and its underground rhizomes hold a significant legacy as medicinal components. With a

traditional application in treating pneumonia, this plant has been utilized in China [4], Thailand [5], and Nepal. In China, the Chinese Pharmacopoeia (2020 edition) documents the medicinal properties of *F. cymosum*, including its ability to clear heat, detoxify, discharge pus, and resolve stasis. Recent research supports these traditional uses by demonstrating *F. cymosum*'s notable anti-tumor, antioxidant, immune-boosting, and antibacterial properties [6]. The beneficial effects of *F. cymosum* are attributed to its rich content of flavonoids and phenolic compounds, including rutin, quercetin, proanthocyanidins, and epicatechin [1]. Presently, over ten Chinese patent medicines utilize *F. cymosum* as a key ingredient, such as "Weimaining capsules," "Jinqiaomai tablets," and "Cough syrup" [7].

The ongoing advancement in research on *F. cymosum* has unveiled its intrinsic value and diverse applications. The surging demand has sparked heightened interest in exploring wild germplasm resources. However, this pursuit not only intensifies ecological degradation but also results in the excessive harvesting of wild germplasm resources, leading to a rapid decline in their populations over a short period [8]. In 1999, China designated *F. cymosum* as a second-class protected plant at the national level. The cultivation of *F. cymosum* is currently nascent, primarily due to its requirement for warm and humid conditions, which restricts suitable planting locations to relatively limited areas. Moreover, the growth of *F. cymosum* is susceptible to variations in temperature and moisture, resulting in variations in quality among different regions. Furthermore, the frequent incidence of elevated temperatures and droughts in recent years has significantly jeopardized farming practices and the existence of wild *F. cymosum*. Drought stands out as one of the prevalent environmental stressors globally, known for its extensive repercussions, substantial harm, and the vast array of regions it impacts [9]. The profound impact of drought on ecosystems and agricultural production results in diminished vegetation growth and low crop yields. This, in turn, threatens national food security and social stability [10]. As global temperatures continue to rise, the frequency and duration of high temperatures and droughts increase. It is anticipated that by the conclusion of the 21st century, the global incidence of severe and pervasive droughts will escalate [11]. Consequently, enhancing the drought tolerance of plants has become imperative, especially for species of high economic or ecological significance like *F. cymosum*, which is presently facing endangerment.

Presently, the predominant strategies for mitigating drought-induced stress in plants consist of cultivating drought-resistant varieties, implementing appropriate irrigation techniques, and enhancing soil quality [12, 13]. These methodologies are known to be time-consuming and labor-intensive. Expressly, the creation of drought-resistant strains necessitates thorough preliminary investigation. Given this obstacle, plant growth-promoting endophytic fungi have surfaced as promising remedies. Endophytic fungi, microorganisms that dwell within plants for prolonged durations, commonly establish mutualistic relationships with their host plants. Those endophytic fungi that benefit plant growth are known as plant growth-promoting endophytic fungi (PGPE) [14]. Endophytic fungi can enhance plant growth by producing hormones, bolstering host stress resistance and facilitating nutrient uptake [15]. Their distribution is extensive and varied, as they have been identified in all terrestrial plants examined thus far. The diversity in species and abundance of endophytic fungi differs among various plants, tissues, and developmental stages. Various factors, including soil type, climate, and the proximity of

neighboring plants, play a crucial role in shaping the composition and diversity of endophytic fungi within plants [14, 16]. The diversity of endophytic fungi significantly contributes to their functional diversity. For example, endophytic fungi that colonize particular plants frequently produce metabolites resembling those of their hosts.

Moreover, those residing in plants located in arid regions typically demonstrate significant resilience to drought. Thus, it is apparent that the attributes of endophytic fungi are closely intertwined with those of their host plants [17, 18]. The interaction between endophytic fungi and their host is intricate and ever-changing. The host offers a protected habitat and vital nutrients to the endophytic fungi, while the fungi, in turn, support the survival of the host plant through diverse mechanisms. These mechanisms encompass hormone secretion, facilitation of nutrient absorption, activation of crucial genes, and enhancement of the host's foundational metabolism [19]. The folate metabolism plays a crucial role in the fundamental metabolic pathways of plants, encompassing processes like DNA synthesis and repair, amino acid metabolism, and methylation reactions. These processes are indispensable for regular cellular functions and life-sustaining activities [20]. Through their interaction with folate metabolism, endophytic fungi are crucial in elevating plant metabolism, enhancing host plant growth and boosting the host's resistance to external stresses [21, 22].

This study examined the diversity of endophytic fungi within *F. cymosum* from diverse habitats and tissue sections. A correlation analysis was conducted to associate endophytic fungi with precipitation levels in the habitat, pinpointing three fungal genera that may assist *F. cymosum* in resilience against drought stress. We successfully isolated 16 fungal strains from the specified genera by employing isolation, purification, and identification procedures. Through conducting in vitro assays to evaluate their growth-promoting and drought-resistance attributes, we ultimately pinpointed three specific strains (JQ_R2, JQ_R14, JQ_L5) exhibiting properties of drought resistance and growth promotion. Subsequent soil inoculation experiments validated these three selected strains' drought-resistant and growth-promoting characteristics. Transcriptomic and metabolomic analyses indicated that JQ_R2 aids *F. cymosum* in resisting drought stress by elevating folate levels and DHFR enzyme activity. Our findings suggest that under arid conditions, *F. cymosum* plants host a diverse array of drought-resistant endophytic fungi, with particular strains enhancing the host plant's ability to combat drought stress by boosting fundamental metabolic processes like folate metabolism.

Materials and Methods

Collection of *F. cymosum* plants from six native habitat sites

In June 2022, wild *F. cymosum* plants were gathered from six diverse native habitat sites and cultivated at the Wild Fagopyrum Germplasm Resource Garden. This garden was collaboratively established by the Institute of Highland Crop Research of Liangshan Prefecture Xichang Academy of Agricultural Sciences and the Chinese Academy of Agricultural Sciences. The garden is located at a longitude of 102°49'E, a latitude of 27°59'N, and an altitude of 2118 meters. The longitude, latitude, and altitude data for the six

indigenous habitat locations of the wild *F. cymosum* plants are provided in Supplementary Table 1. A total of 30 *F. cymosum* plants were collected from these sites, with five plants, comprising roots, stems, and leaves, gathered from each location for analysis in the laboratory.

Extraction of total DNA from *F. cymosum* and high-throughput sequencing of ITS sequences

Genomic DNA was extracted using the OMEGA Soil DNA Kit (Omega Bio-Tek, Norcross, GA, United States) and stored at -20°C for subsequent analysis. DNA quality was evaluated using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis. The fungal internal transcribed spacer (ITS rDNA) was amplified with primers ITS1F and ITS2R (Supplementary Table 2). The PCR amplicons obtained were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China), and their quantity was determined using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After quantification of each sample, the amplicons were combined in equal proportions and then underwent pair-end sequencing with 2 × 250 bp on the Illumina NovaSeq platform using the NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd. in Shanghai, China. The raw DNA sequence information has been deposited in the National Center for Biotechnology Information's Sequence Read Archive database under the BioProject accession number PRJNA1135529.

Acquisition of precipitation factors from the six native habitat sites

Historical climate data were obtained from the WorldClim website (version 2.1, released in January 2020) covering 1970 to 2000. The bioclimatic data on the historical climate data page typically have a 30 arc-second resolution. After download and extraction, 19 files with a .tif extension were acquired, each corresponding to a distinct BIO factor (supplementary table 3). The WorldClim website can be accessed at <https://www.worldclim.org/data/worldclim21.html>. Launch the ArcMap software and import the WorldClim dataset to commence the analysis. Subsequently, upload the latitude and longitude coordinates of the six designated sampling locations. Generate a new layer and export the sample information file encompassing the geographical attribute data. This file includes the 19 BIO factor datasets linked to the native habitat sites, featuring BIO12 to BIO19, representing the precipitation-related variables.

Isolation, purification, and identification of endophytic fungi from the roots, stems, and leaves of *F. cymosum*

Within 12 hours of sample collection, the materials were washed with sterile water and surface-sterilized, following the method outlined by Coombs and Franco [23] with some adjustments. The procedure involved soaking root, stem, and leaf tissues in 75% ethanol for 60 seconds on a sterile workbench, then immersing them in 2.5% sodium hypochlorite for 5 minutes and finally rinsing them 5–6 times with sterile water. After air-drying, the samples were trimmed into approximately 1 cm × 1 cm

squares using a sterile blade and positioned on autoclaved potato dextrose agar (PDA) plates enhanced with 0.01% ampicillin. The trimmed edges were placed in direct contact with the agar surface. Subsequently, the plates were incubated in darkness at 28°C for 3–4 days to isolate individual fungal strains by transferring the tips of the hyphae extending from the sample edges to fresh plates. The process was iterated until a solitary hyphal strain was isolated. The refined fungi were subsequently introduced onto a PDA solid slant medium. Following substantial mycelial proliferation, they were preserved at 4°C in a refrigerator and reactivated quarterly.

The identification of endophytic fungi relies primarily on molecular biology, complemented by morphological identification. The latter entails observing and documenting the fungal hyphae's growth rate on plates, the morphology of both colonies and hyphae and any pigment production on the plates. In molecular biology identification, fungal DNA is extracted using the CTAB method [24]. The 5.8S rDNA sequence of fungi is amplified using universal fungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') based on the ITS sequence. Following confirmation through agarose gel electrophoresis that the fragment size falls within the range of 500 bp to 750 bp, the PCR products are extracted from the gel according to the TianGen Gel Extraction Kit guidelines. The DNA samples are forwarded to Youkang Biotechnology Co., Ltd. for sequencing after purification. Subsequently, the acquired DNA sequences undergo comparison against entries in the NCBI database employing BLAST. The ten sequences exhibiting the most significant similarity are retrieved. A phylogenetic tree is then constructed utilizing the neighbor-joining method within the MEGA 7.0 software platform.

In vitro detection of IAA production and drought resistance of endophytic fungi

The amount of IAA in the fungal strain's fermentation broth was quantified using the Salkowski colorimetric method following the protocol outlined by Glickmann and Dessaux [25]. This involved the preparation of various standard IAA solutions alongside the supernatant derived from the endophytic fungal fermentation broth. Equal volumes of the standard solutions or samples were combined with the Salkowski colorimetric solution, while a mixture of PDB culture medium and Salkowski reagent served as the blank control. Subsequently, all samples were incubated in a dark chamber at room temperature for 30 minutes. The absorbance was measured at OD530, and the IAA content was calculated using the IAA standard curve. Following the methodology established by Zhujiang-Kang's team [26], PDA solid medium with 8% and 12% PEG was formulated to select drought-tolerant endophytic fungal strains. In brief, a liquid containing the designated PEG concentration was poured onto solidified PDA plates and left covered overnight for equilibration. Fungal blocks, each with a diameter of 5 mm, were then inoculated onto PDA plates with varying concentrations of PEG6000 (0%, 8%, and 12%). Subsequently, the daily measurements of colony diameter were recorded to create growth curves. A strain is considered drought-tolerant if it exhibits normal or accelerated growth on plates containing PEG6000.

Inoculation and detection of endophytic fungi colonization in *F. cymosum*

Inoculate fungal mycelia into 300 mL of Potato Dextrose Broth (PDB) liquid medium and incubate in darkness at 28°C with shaking at 180 rotations per minute for five days. Subsequently, the mycelia was harvested through centrifugation at 12,000 rotations per minute, followed by homogenization in sterile water using a blender to produce a slurry with a 0.1 g/mL concentration. In this study, one-month-old seedlings of *F. cymosum* were inoculated at the root zone with a mycelial slurry (3 mL per plant) by root drenching. The injection was repeated weekly for a total of five times. The roots were stained to determine the presence of fungal colonization and evaluate colonization. Two days after the initial injection, the fresh roots were digested in a 10% KOH (m/v) solution at 90°C for 2 hours, with the KOH solution being replaced every 30 minutes. Treat the roots with 2% hydrochloric acid (HCl v/v) for 10 minutes and three washes with sterile water. Incubate the roots at 37°C for 30 minutes in a solution containing 5.0 µg/mL of wheat germ agglutinin Alexa Fluor 488 conjugates (WGA488). Utilize a fluorescence microscope (OLYMPUS, BX53) to observe fungal colonization within the plant roots [27].

Application of drought treatment

After confirming the colonization of fungal strains through fluorescence staining, proceed to evenly transplant *F. cymosum* seedlings, both inoculated and non-inoculated, into planting boxes. Each box, measuring 40 cm in length, 30 cm in width, and 36 cm in height, will accommodate six seedlings. JIFFY pure peat soil should be utilized for planting purposes. In the context of water treatment in this study, the drought moisture (DM) treatment regimen entails watering the plants once every two weeks, while the average moisture (NM) treatment group receives weekly watering sessions, with each plant receiving 300 mL per watering. In the study, *F. cymosum* seedlings were cultivated hydroponically, where drought stress was simulated using a Hoagland nutrient solution supplemented with 15% PEG6000. In contrast, the control group received a standard Hoagland nutrient solution without PEG6000. The experiment occurred in a plant greenhouse cultivation room set at 25°C, with a light/dark cycle of 16 and 8 hours and a relative humidity of 60%.

Assessment of Phenotypic and Physiological Parameters in *F. cymosum*

The phenotypic traits of soil-cultivated *F. cymosum* plants were assessed by measuring plant height, petiole length, and leaf dimensions (length and width). The diameter of the main stem was determined using a vernier caliper, while the count of central stem nodes was conducted directly. Physiological indicators were assessed through the measurement of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) enzyme activities, as outlined by Nahakpam and Shah [28]. The levels of glutathione (GSH), proline, and malondialdehyde (MDA) were determined using the procedures established by Alves et al. [29]. Furthermore, phenylalanine ammonia-lyase (PAL) activity and soluble sugar content were evaluated following the protocols in the study by Zhang et al. [30].

Transcriptomic and metabolomic analysis with real-time quantitative PCR

Two experimental groups were established under normal and drought water conditions: one group was inoculated with JQ_R2, while the other remained non-inoculated. Each experimental group comprised three independent biological replicates designated for RNA-seq analysis. Following RNA extraction, the quality and integrity of the RNA samples were evaluated utilizing a NanoPhotometer spectrophotometer and an Agilent 2100 bioanalyzer. The cDNA libraries were constructed using the NEBNext Ultra™ RNA Library Prep Kit for Illumina. Following library preparation, the initial quantification was carried out with a Qubit 2.0 Fluorometer, and the library insert size was assessed using an Agilent 2100 Bioanalyzer to verify the quality of the libraries. The libraries that met the proficiency criteria were subsequently sequenced using Illumina sequencing technology, which operates on the Sequencing by Synthesis principle [31]. After performing quality control on the raw reads, clean data were obtained. Subsequently, the paired-end clean reads underwent alignment to the reference genome of *F. cymosum*, which had been previously sequenced and spliced by our team [32] using HISAT2 v2.0.5. Differential expression analysis between the two comparison groups was done using DESeq2 software (v1.16.1) [33], with p-values adjusted using the Benjamini & Hochberg method. Significant differential expression was determined by an adjusted p-value ($p < 0.05$) and $|\log_2\text{foldchange}| > 1$. The study conducted enrichment analyses of differentially expressed genes (DEGs) using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. The clusterProfiler package in R (version 3.4.4) was utilized for these analyses. Enrichment of GO and KEGG terms was deemed significant if the adjusted p-value was less than 0.05. The raw data can be accessed in the NCBI Sequence Read Archive under the accession number PRJNA1135705.

The RNA-seq data and expression levels of various genes were validated through qRT-PCR analysis conducted with the Archimed X series real-time fluorescence quantitative PCR instrument. The housekeeping genes, *Actin* and *Box*, were employed to normalize target gene expressions [34]. Li et al. [35] described that the quantitative PCR primers were developed using Primer Premier 5 software, with detailed primer sequence information in Supplementary Table 4. The optimal annealing temperature for each primer pair was determined through a gradient program during the initial testing. To guarantee the reproducibility of the qRT-PCR reactions, every sample underwent analysis in three technical replicates and three biological replicates.

The samples utilized for metabolomic analysis were in alignment with those utilized for transcriptomic analysis. Following grinding in liquid nitrogen, the samples were subjected to extraction with 80% methanol, followed by centrifugation to collect the supernatant for subsequent analysis employing liquid chromatography – mass spectrometry (LC-MS). The LC-MS analysis was conducted using a Q Exactive™ HF/Q Exactive™ HF-X mass spectrometer (Thermo Fisher, Germany) and a Vanquish UHPLC chromatograph (Thermo Fisher, Germany). A Hypersil Gold column (100×2.1 mm, 1.9 μm, Thermo Fisher, USA) was employed in chromatography, set at a column temperature of 40°C and with a flow rate of 0.2 mL/min. The mobile phases consisted of 0.1% formic acid for phase A and methanol for phase B, with the specific chromatographic gradient elution program outlined in Supplementary Table 5. Mass spectrometry conditions were set as follows: Spray Voltage at 3.5 kV, Sheath gas flow rate at 35 psi, Aux Gas flow rate at 10 L/min, Capillary Temp at 320°C, S-lens RF level at 60, Aux gas heater temp at

350°C, Polarity included positive and negative modes, and MS/MS secondary scanning for data-dependent scans. The raw data files (.raw) underwent processing through CD3.3 software, which included peak extraction, peak area quantification, and matching predicted molecular formulas with databases such as mzCloud (<https://www.mzcloud.org/>), mzVault, and Masslist. Ultimately, this process facilitated the identification and relative quantification of metabolites. The data processing was performed on a Linux operating system (CentOS version 6.6) using R and Python software. The annotation of identified metabolites was carried out by referencing the KEGG database (<https://www.genome.jp/kegg/pathway.html>), HMDB database (<https://hmdb.ca/metabolites>), and LIPIDMaps database (<http://www.lipidmaps.org/>). The metabolite data has been deposited in MetaboLights (<https://www.ebi.ac.uk/metabolights/>) with the identifier MTBLS10685.

Temporary transformation of detached leaves and the foliar application of folic acid on plants

The coding sequence (CDS) of the *FcDHFR-TS* gene was cloned into the pCAMBIA1300-UBQ10-GFP overexpression vector using seamless cloning and subsequently transferred into *Agrobacterium tumefaciens* (GV3101). *Agrobacterium* was transformed into the leaves of *F. cymosum* plants using a plant in vivo transformation device (vacuum, 6 KPa, 5 min), with an empty vector employed as the control. To evaluate drought tolerance, the water loss rate of the transformed leaves was measured two days post-transformation [36].

Folic Acid (FA) was procured from Shanghai Yuanye Bio-Technology Co., Ltd. Following the method described by Ibrahim et al. [37], a control group with 0 FA (foliar spray with distilled water) and an experimental group with 150 µM FA were established. *F. cymosum* plants were cultivated hydroponically under drought-simulating conditions utilizing 15% PEG6000. *F. cymosum* plants were subjected to foliar sprays of either distilled water or 150 µM FA starting ten days before the drought initiation. Each plant was administered 3 ml of the solution daily for ten consecutive days. Due to FA's high photostability in sunlight, the treatments were administered in the evening (before the plant room lights off) following the procedure outlined in the reference [38]. After the drought commencement, the phenotypic alterations in *F. cymosum* plants were consistently monitored.

Determination of folic acid content and DHFR enzyme activity

The double antibody sandwich method assessed plants' folic acid content and DHFR enzyme activity. Initially, microplates were coated with purified plant folic acid or dihydrofolate reductase antibodies to establish a solid-phase antibody. Following the coating of microplates with monoclonal antibodies, folic acid or dihydrofolate reductase was introduced, along with HRP-labeled folic acid or dihydrofolate reductase antibodies, resulting in the formation of an antibody-antigen-enzyme-labeled antibody complex. Subsequently, after a thorough washing step, the substrate TMB was added to initiate color development. The HRP enzyme catalyzed TMB, causing it to shift from blue to the eventual yellow hue under acidic conditions. The color intensity was directly correlated to folic acid content or dihydrofolate

reductase in the sample. The absorbance (OD value) at 450 nm was determined using a microplate reader, and the concentration of plant folic acid or dihydrofolate reductase activity in the samples was calculated by referencing a standard curve.

Statistical analysis

The bar graphs in the study were generated using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA). Mean values and standard errors (SE) were computed, and the treatment variations were assessed through ANOVA analysis (Duncan's multiple range test) or Student's t-tests. A significance threshold of $p < 0.05$ was utilized to identify noteworthy distinctions between groups.

Results

The richness and diversity of endophytic fungi in *F. cymosum* vary across habitats and tissue compartments.

The southwestern region of China, including Sichuan, Yunnan, Guizhou, and Tibet, is renowned as the primary center of origin, distribution, and diversification of buckwheat (genus *Fagopyrum*) [39]. To assess the diversity and variability of endophytic fungi in wild *F. cymosum* across six native habitat sites in four regions (Fig. 1A), high-throughput sequencing of the ITS region was conducted. A total of 4,052,907 compelling reads and 4,052,889 high-quality sequences were acquired from 18 *F. cymosum* tissue samples, leading to the detection of 1,122 operational taxonomic units (OTUs). The sequence lengths varied from 182 to 351 base pairs. The smoothness of the sparse curves suggested that sequencing depth impacted the observed diversity in the samples. As the sequencing depth increased, the curve representing the number of OTUs in the 18 samples leveled off (Supplementary Fig. S1G). This suggests that the sequencing outcomes successfully captured the existing diversity in the sample, and additional increments in sequencing depth did not unveil new OTUs. The Venn diagram of OTUs illustrates that endophytic fungi of *F. cymosum* from six distinct habitats exhibit 46 shared OTUs, with YL, LZ, HL, NH, KM, and HZ individually harboring 147, 237, 296, 111, 99, and 186 unique OTUs, as depicted in Fig. 1B. Within the various tissue groups, roots, stems, and leaves contain 418, 356, and 267 unique OTUs, respectively, while they share 378 OTUs among them (Fig. 1C).

The species abundance classification plot of the top 10 phyla illustrates that *Ascomycota* is the predominant phylum among the endophytic fungi of *F. cymosum*, with *Basidiomycota* following closely behind (Supplementary Fig. S1A, D). In terms of class, *Dothideomycetes*, *Sordariomycetes*, and *Agaricomycetes* constitute the predominant classes among endophytic fungi, as depicted in Supplementary Fig. S1B, E. Regarding the order level, *Capnodiales*, *Glomerellales*, and *Pleosporales* emerge as the top three abundant orders, as shown in Supplementary Fig. S1C, F. Endophytic fungi of wild *F. cymosum* from six habitat sites display notable variations in genus-level abundance. For example, *Septoria* fungi comprise 25.96% of the HL habitat samples, whereas they only comprise 2.01% of the LZ samples. In contrast, *Colletotrichum* fungi account for 37.99% of LZ but less than 2% in NH samples (Fig. 1D). Distinct variations in genus-level fungi are evident among various tissue compartments.

Notably, *Ilyonectria* is the most abundant genus in roots, accounting for 14.43%, while its presence is less than 4% when considering stems and leaves together. In stems, *Vishniacozyma* dominates with 16.04%, whereas *Septoria* prevails in leaves at 28.68% (Fig. 1E).

The alpha diversity of the 18 samples was evaluated utilizing community richness (Chao1 index, Observed species), coverage (Good coverage index), diversity (Shannon index, Simpson index), and evenness (Pielou's index) metrics, which are outlined in Supplementary Table S6. Among the samples from various tissue compartments (roots, stems, leaves), the stem samples demonstrated the highest richness and diversity of endophytic fungi, along with the most evenly distributed fungal communities, as shown in Fig. 1F, G. HL displayed higher Chao1 and Observed species indices among the six distinct habitat sites, suggesting more extraordinary richness compared to HZ, which had the lowest values. Regarding community diversity, both HL and NH exhibited higher diversity than ZL. The robust fungal sequencing coverage, with Good coverage values surpassing 99.8% across all samples, accurately portrays the fungal communities within the samples. Furthermore, there were no substantial differences in evenness among the samples.

By employing the Bray-Curtis distance algorithm for ANOSIM analysis, clustering was conducted on the endophytic fungal communities of *F. cymosum* across six distinct habitats. The outcomes revealed notable discrepancies in species composition among sample groups in contrast to within-group variations ($R = 0.383$, $P = 0.01$), emphasizing habitat-specific differences in *F. cymosum*, as illustrated in Supplementary Fig. S1H. Likewise, ANOSIM analysis of distinct plant tissues indicated that the disparities between groups were significantly more pronounced than the differences within groups ($R = 0.223$, $P = 0.001$) (Supplementary Fig. S1I). Furthermore, PCoA analysis unveiled variations in endophytic fungal communities within *F. cymosum* samples, with PCo1 accounting for 13.1% of the variance and PCo2 explaining 10.9% (Fig. 1H, I). Notably, *F. cymosum* from Linzhi City in Tibet displayed significant distinctions compared to other habitats. KM and NH exhibited a closer relationship, while HZ, HL, and YL showed more dispersion, signifying substantial variations in fungal communities among roots, stems, and leaves. Notably, the fungi in stems and leaves demonstrated more remarkable similarity to those in roots.

Endophytic fungi at the genus level of *Fusarium*, *Colletotrichum*, and *Ceratobasidium* may be involved in drought tolerance of *F. cymosum*

Endophytic fungi within plants have engaged in extended co-evolution with their hosts, frequently establishing mutualistic associations. The host offers a "habitat" and nourishment to the endophytic fungi, which reciprocate by aiding the host in improved uptake of external nutrients like nitrogen, phosphorus, and other inorganic elements. Furthermore, these endophytic fungi elevate the host's photosynthetic efficiency and fortify its resilience against diverse abiotic and biotic stresses, encompassing drought, salinity, and instances of pest and disease attacks [40, 41]. Thus, our hypothesis suggests the presence of endophytic fungal strains in *F. cymosum* that stimulate growth and enhance the plant's resistance to external stresses. Each habitat where *F. cymosum* samples are found exhibits

unique ecological climates. We referenced the WordClim database to analyze the climatic characteristics of six native habitat sites, revealing notable variations in precipitation levels (Supplementary Table S7), notably during the driest quarter and the driest month. Notably, Linzhi city in Tibet experiences the lowest annual precipitation, while Huili County in Sichuan records the highest levels. In our quest to identify drought-resistant fungal strains in *F. cymosum*, we pinpointed 18 common genera of endophytic fungi across 18 sample groups (Fig. 2A). Subsequently, we conducted a Pearson correlation analysis between these shared genera and eight precipitation factors (Supplementary Fig. S2). The findings revealed that the genera *Fusarium*, *Colletotrichum*, and *Ceratobasidium* exhibited negative correlations with precipitation variables, notably annual precipitation, driest month precipitation, and driest quarter precipitation (Fig. 2B). This suggests that these three genera of endophytic fungi are predominantly present in *F. cymosum* plants from regions with low precipitation levels. In conclusion, it is postulated that these genera may play a role in the drought resistance of *F. cymosum*.

For the isolation of endophytic fungi belonging to the genera *Fusarium*, *Colletotrichum*, and *Ceratobasidium* from *F. cymosum*, we utilized the tissue block isolation technique and successfully isolated 59 endophytic fungal strains from the roots, stems, and leaves of *F. cymosum*. By employing morphological and molecular biological analyses, a total of 37 species were identified (Supplementary Table 8). The growth morphology of the 37 endophytic fungi on plates is illustrated in Supplementary Fig. S3. Through phylogenetic analysis, we identified 13 *Fusarium* strains (labeled JQ_R1, JQ_R2, JQ_R3, JQ_R5, JQ_R6, JQ_R7, JQ_R8, JQ_R9, JQ_S1, JQ_S2, JQ_S3, JQ_L1, JQ_RS1), 2 *Colletotrichum* strains (labeled JQ_L4, JQ_L5), and 1 *Ceratobasidium* strain (labeled JQ_R14) among these 37 isolates (Fig. 2C). The viability of the strains on drought plates was documented to assess their capacity for average growth under drought circumstances, and survival curves were subsequently generated (Supplementary Fig. S4). Specifically, twelve strains exhibited average growth on 8% PEG6000 plates, while eleven strains displayed average growth on 12% PEG6000 plates. Next, the strains' capacity to secrete indole-3-acetic acid (IAA) in vitro was evaluated to determine their potential for promoting host growth (Supplementary Fig. S5). Among them, eight strains exhibited notably high levels of IAA production. Following these findings, three strains demonstrating robust capabilities in drought resistance and growth promotion were identified: JQ_R2 (*Fusarium* sp.), JQ_R14 (*Ceratobasidium* sp.), and JQ_L5 (*Colletotrichum* sp.). The growth profiles of JQ_R2, JQ_R14, and JQ_L5 are depicted in Fig. 2D, E, and F, respectively, while their levels of IAA secretion are detailed in Fig. 2G. The ensuing investigation will examine the colonization of these three fungi within *F. cymosum* and their impacts on plant growth.

JQ_R2, JQ_R14, and JQ_L5 demonstrate growth-promoting and drought-resistant effects upon inhabiting *F. cymosum*.

The process of fungal colonization from external sources to the interior of plants is influenced by various factors, with the primary influence stemming from the fungi themselves. Different fungal species demonstrate varying efficiency in colonization and differences in preferred colonization sites and modes [42–44]. Despite being isolated from *F. cymosum*, the colonization efficiency and post-colonization morphology of JQ_R2, JQ_R14, and JQ_L5 remain obscure. To address this gap, we employed the

fluorescent dye WGA-AF488 for fungi labeling within the plant, enabling the observation of their colonization efficiency and morphology. The fluorescence staining analysis revealed that all three strains exhibited the capability to colonize the roots of *F. cymosum* from exogenous sources (Fig. 3A). Among the strains, JQ_R14 displayed the most significant and dense colonization, predominantly through hyphae and sclerotia within root cells. The hyphae of JQ_R14 were thick, densely packed, and featured well-defined septa. Sclerotia were interspersed among the hyphae, forming elliptical structures. Similarly, JQ_R2 also exhibited colonization through hyphae and sclerotia, although its hyphae were thin, sparsely distributed, and lacked septa. The sclerotia predominantly exhibited elongated shapes. In contrast to the two other strains, JQ_L5 colonized exclusively through thin, curved, and extensively branched hyphae. Furthermore, small protrusions, likely representing newly divided short hyphae, were frequently noticeable along the hyphae.

The *F. cymosum* plants cultivated in the soil to mimic field conditions revealed that under NM circumstances, the inoculation with fungal strains notably enhanced plant growth (Fig. 3B). Mainly, substantial improvements were evident in plant height and central stem thickness, displaying significant increments post-inoculation with JQ_R2, JQ_R14, and JQ_L5 (Fig. 3C). Moreover, there was a significant increase in the number of central stem nodes and leaf size. Specifically under NM conditions, the inoculation with JQ_R2 notably boosted the activities of POD, SOD, and CAT enzymes. Similarly, injection with JQ_R14 resulted in significant enhancements in the activities of POD and SOD, whereas immunization with JQ_L5 markedly elevated the activities of POD and CAT. Moreover, the levels of proline and soluble sugars surged post-fungal inoculation, with JQ_R2 and JQ_L5 amplifying the GSH content, although the PAL content remained unaltered. In DM conditions, the plant phenotype (including height, stem thickness, water content, etc.) exhibited a substantial decline when contrasted with NM conditions, whereas enzyme activities and osmolyte contents showed a marked increase. In DM conditions, inoculated plants exhibited elevated plant height, stem and leaf water content, and main stem node numbers compared to non-inoculated plants. Moreover, there was a significant increase in the activities of POD, SOD, and CAT enzymes, alongside higher osmolyte levels and reduced MDA content (Fig. 3C, Supplementary Fig. S6).

To model abrupt extreme drought conditions, we cultivated *F. cymosum* seedlings hydroponically and supplemented the nutrient solution with varying concentrations of PEG6000 to replicate severe drought stress. To ascertain the optimal concentration of PEG6000 for drought simulation, we subjected one-week-old *F. cymosum* seedlings to pre-treatment with PEG6000 concentrations of 0%, 5%, 10%, 15%, and 20%. We monitored leaf curling hourly, documenting our observations through photography and recording. The condition of the seedlings was assessed at two distinct time points: initially, at the commencement of leaf water loss and curling, and subsequently at a stage of severe water loss and curling (Supplementary Fig. S7). The findings revealed that leaf curling was induced by 5% and 10% PEG6000 at 56 and 48 hours, respectively, with severe water loss observed at 80 and 72 hours. Conversely, exposure to 20% PEG6000 resulted in leaf curling within 1 hour, followed by severe water loss leading to near-fatality after 5 hours. On the other hand, with 15% PEG6000, leaf curling manifested swiftly at 2 hours and endured for a significant period, enabling the plants to react and withstand, with

severe water loss only manifesting after 56 hours. Consequently, 15% PEG6000 was chosen as the concentration for simulating drought stress.

Phenotypically, under 0% PEG6000 conditions, the inoculation with fungal strains led to increased leaf emergence in *F. cymosum* seedlings, particularly noticeable in plants treated with JQ_R2. Following three days of exposure to 15% PEG6000, non-inoculated (NI) plants displayed severe drought symptoms characterized by near-complete leaf curling. Fungi-inoculated plants with JQ_R14 and JQ_L5 exhibited reduced drought symptoms, with the curling confined to older leaves, while most new leaves remained unaffected. In contrast, plants treated with JQ_R2 displayed minimal leaf chlorosis and virtually no leaf curling, sustaining regular growth patterns (Fig. 3D). Subsequently, a range of enzyme activities and substance contents were assessed in the hydroponically grown seedlings (Fig. 3E, Supplementary Fig. S7). Without PEG6000 (0% conditions), the injection with JQ_R2 markedly raised the levels of PAL, soluble sugars, and GSH. Conversely, at 15% PEG6000 conditions, notable alterations included a significant surge in POD enzyme activity and a marked reduction in MDA content post-fungal vaccination, particularly with JQ_R2. These patterns aligned with the trends observed in *F. cymosum* plants cultivated in soil.

A combined transcriptome and metabolome analysis revealed that folate metabolism might be involved in alleviating drought stress in *F. cymosum* by JQ_R2.

Given the superior drought tolerance exhibited by *F. cymosum* inoculated with JQ_R2 in prior experiments, we concentrated on evaluating the efficacy of the JQ_R2 strain in subsequent validations. To meticulously investigate the morphology of JQ_R2 hyphae colonizing the roots of *F. cymosum*, scanning electron microscopy was employed for a detailed examination of the hyphal presence on the root surface (Fig. 4A). In the absence of fungal inoculation, the root surface displayed solely disorganized root hairs and surface grooves of differing depths. Conversely, upon inoculation with the JQ_R2 strain, at low magnification ($\times 300$), many hyphae were detected intertwined with root hairs, with some hyphae tightly adhering to the grooves on the surface. The hyphae presented as smooth, elongated rods, with specific hyphal tips swelling to create spherical sporangia structures that released spores upon rupture. When examined at high magnification ($\times 2000$), the attachment of hyphae to the root surface, sporangial structure, and the morphology of certain tiny spores were distinctly observable.

To elucidate the molecular mechanisms through which JQ_R2 mitigates drought stress in *F. cymosum*, we examined the transcriptome data from *F. cymosum* leaves. Following quality filtering, a cumulative data of 84.32 Gb was sourced from 12 leaf samples of *F. cymosum*. Each sample provided over 6 GB of data, reflecting a Q20 rate of $\geq 99.385\%$ and a Q30 rate of $\geq 97.826\%$ (Supplementary Table S9). The quality-approved clean data reads were aligned to the reference genome utilizing the HISAT2 software, followed by an alignment assessment through the Qualimap RNA-seq pipeline. The comprehensive mapping rate across the 12 samples exceeded 89.4%, signifying the successful alignment of most sequencing reads to the reference genome. Furthermore, the exonic mapping rate for all samples exceeded 70%, signifying robust data quality and specificity. Moreover, 5'-3' bias values surpassing 1

indicated that the RNA samples were intact and not degraded (Supplementary Table S10). In summary, the transcriptome data are deemed precise and dependable, making them suitable for further analyses. The figure in Fig. 4B illustrates the differentially expressed genes (DEGs) count in the four comparison groups, based on $|\log_2 \text{fold change}| \geq 1$ and $p \leq 0.05$. The comparison between CK and DCK revealed the most significant number of DEGs, with 185 genes upregulated and 138 downregulated. Conversely, the CK vs. R2 comparison exhibited the lowest count of DEGs, with 30 genes upregulated and 48 downregulated. A Venn diagram, depicted in Fig. 4C, displayed the distribution of DEGs across the four comparison groups, highlighting that the CK vs. DCK group possessed the highest number of unique DEGs (250), followed by the R2 vs. DR2 group with 206 exclusive DEGs. Only five common DEGs were identified between the two experimental groups, suggesting that the injection with JQ_R2 significantly influenced the drought response of *F. cymosum* plants. Notably, no DEGs were shared between the experimental groups subjected to standard and drought conditions. The number of DEGs observed under drought conditions with fungal inoculation exceeded those identified under normal conditions, indicating a more pronounced impact of JQ_R2 on *F. cymosum* plants under drought stress. We conducted a Gene Ontology (GO) enrichment analysis to ascertain the roles of the identified DEGs, as depicted in Supplementary Fig. S8. The analysis revealed enrichment of GO terms across three principal functional categories: molecular function (MF), cellular component (CC), and biological process (BP). Within the CK vs. DCK group, the DEGs were predominantly enriched in the MF and BP categories, with a notable enrichment identified in transferase activity, specifically in transferring glycosyl groups. The CK vs. R2 comparison group exhibited enrichment across the MF, CC, and BP categories, particularly emphasizing ribosome and cytosolic ribosome in the CC category. Conversely, in the DCK vs. DR2 group, the DEGs were predominantly enriched in the BP category, with only a few in the CC category. Notably, cellular response to acid chemicals was the most enriched GO term within the BP category. In the R2 vs. DR2 comparison group, most DEGs were enriched in the BP category, with a notable enrichment observed in the organic hydroxy compound metabolic process. Furthermore, the KEGG enrichment analysis offered comprehensive insights into the metabolic and signal transduction pathways influenced by the DEGs. Within the CK vs. DCK experimental group, differentially expressed genes (DEGs) were predominantly linked to plant hormone signal transduction and the MAPK signaling pathway. Conversely, in the CK vs. R2 comparison group, DEGs were primarily enriched in the flavone and flavonol biosynthesis and the phenylpropanoid biosynthesis pathways. In the R2 vs. DR2 experimental group, DEGs were predominantly enriched in fatty acid degradation and several amino acid metabolic pathways, including tyrosine, cysteine, and methionine metabolism (Supplementary Fig. S9). In the DCK vs. DR2 comparison group, DEGs were significantly associated with carbon metabolism and the metabolism of tryptophan, glutathione, glycine, and serine (Fig. 4D).

Transcriptomic analysis facilitates the identification of differentially expressed genes, whose upregulation or downregulation can influence the levels of downstream substances, consequently eliciting diverse plant responses. Nevertheless, gene expression regulation and metabolic regulation are intricate processes influenced by multiple layers and factors. It is important to note that alterations in gene expression levels do not always correlate directly with changes in the content of downstream

substances. To address this uncertainty and precisely elucidate the pathways involved in regulating drought tolerance by JQ_R2 in *F. cymosum*, we performed untargeted metabolomic analysis on the leaves of four experimental groups. Compared to the control group, the OPLS-DA analysis unveiled notable biochemical alterations in the leaves of plants treated with the JQ_R2 strain (Supplementary Fig. S10). This suggests that VIP analysis is a valuable tool for metabolite screening. The differential accumulation of metabolites (DAMs) in the four experimental comparison groups was determined using $|\log_2 \text{fold change}| > 1$ and $\text{raw. pval} < 0.05$, as shown in Fig. 4E. The CK vs. DCK comparison group exhibited the highest count of differentially accumulated metabolites (DAMs), comprising 47 down-regulated and 86 up-regulated metabolites. Subsequently, the CK vs. R2 comparison group identified 93 DAMs, while the DCK vs. DR2 and R2 vs. DR2 experimental groups revealed 70 and 55 DAMs, respectively. A Venn diagram illustrating the differential metabolites across the four experimental groups was presented (Fig. 4F). It revealed that the CK vs. DCK and CK vs. R2 comparison groups shared 33 common DAMs. Conversely, the CK vs. DCK and R2 vs. DR2 groups exhibited only 4 shared DAMs, while the CK vs. DCK group contained 64 unique DAMs, indicative of the substantial influence of JQ_R2 strain inoculation on metabolite secretion in drought-stressed plants. Following this, KEGG enrichment analysis was conducted on the differentially accumulated metabolites (DAMs). In the CK vs. DCK experimental group, DAMs were predominantly enriched in nucleotide metabolism and the synthesis and degradation of flavonoids. Similarly, in the CK vs. R2 comparison group, the metabolites were chiefly enriched in purine, nucleotide, and amino acid metabolism. Moreover, in the R2 vs. DR2 comparison group, the DAMs were primarily enriched in the biosynthesis and metabolism of phenylpropanoids, flavonoids, and amino acids (Supplementary Fig. S11). Finally, in the DCK vs. DR2 comparison group, the DAMs were predominantly enriched in the biosynthesis and metabolism of various amino acids, including cysteine, methionine, arginine, and proline (Fig. 4G).

Utilizing the outcomes of the multi-omics analysis, our objective was to elucidate the mechanisms by which JQ_R2 aids *F. cymosum* plants in mitigating drought stress. In the previous KEGG enrichment analysis, our attention was directed toward the findings of the DCK vs. DR2 comparison group. Given that the DEGs were predominantly enriched in carbon metabolism and amino acid metabolism, and the DAMs were also chiefly enriched in amino acid synthesis and metabolism, our attention was drawn to a metabolite closely associated with both carbon and amino acid metabolism—folate. The folate content of *F. cymosum* plants exhibited a significant decrease under drought stress; however, subsequent inoculation with the JQ_R2 strain restored the folate levels to normal (Fig. 4H). Folate synthesis and utilization are intricately linked to dihydrofolate reductase (DHFR), an enzyme that converts dihydrofolate to folate. Particularly essential is the role of folate in its active form as tetrahydrofolate, a conversion process facilitated by DHFR. In our transcriptome analysis, we noted a significant decrease in the expression of dihydrofolate reductase-thymidylate synthase (DHFR-TS) in *F. cymosum* plants under drought stress. Remarkably, the expression levels of *DHFR-TS* markedly increased following inoculation with JQ_R2, aligning with the observed pattern in folate content (Fig. 4H). Therefore, we postulate that the JQ_R2 strain may assist *F. cymosum* plants in mitigating drought stress via the folate metabolic pathway.

Additionally, we validated the precision of the transcriptome data using real-time quantitative PCR. The expression levels of *DHFR-TS* in *F. cymosum* leaves corresponded with the transcriptome findings, indicating decreased expression post-drought and a substantial increase following JQ_R2 inoculation. Investigating the tissue-specific expression of the *DHFR-TS* gene, we assessed its levels in roots and stems. Our findings revealed a notable decrease in *DHFR-TS* expression in roots but an increase in stems under drought conditions. Interestingly, post-inoculation with JQ_R2, the expression levels of *DHFR-TS* in both roots and stems rose during drought stress. Besides *DHFR-TS*, other pivotal genes in the folate synthesis pathway, including *ADCS*, *ADCL*, *GTPCHI*, *DHNA*, and *DHFS* [45], are crucial in folate synthesis. Consequently, we evaluated the relative expression levels of these five genes (Fig. 4I). A significant trend emerged as the expression levels of these genes substantially increased in root tissues under drought conditions and post-inoculation with JQ_R2. In leaf tissues, apart from a reduction in *ADCS* expression and the absence of significant change in *GTPCHI* expression, the expression levels of the remaining genes also notably rose under drought conditions and following fungal inoculation. Nevertheless, the alterations in stem tissues displayed inconsistency. For example, *ADCS* expression increased post-inoculation under normal conditions but decreased significantly after inoculation under drought conditions. A typical pattern emerged where the expression levels of these folate synthesis-related genes in stem tissues rose under drought conditions without fungal inoculation.

The JQ_R2 strain may aid host plants in resisting drought stress by elevating folate content and boosting DHFR enzyme activity in *F. cymosum*

To investigate the potential of folate in aiding *F. cymosum* plants in withstanding drought stress, we conducted an in vitro folate spraying experiment. Following slight adjustments to the protocols described by Khan et al. [46] and Ibrahim et al. [37], we utilized foliar spraying of 150 μ M folate for the experimental group (with distilled water spraying as the control) and monitored the growth response of *F. cymosum* plants pre- and post-drought stress. Our observation revealed that *F. cymosum* plants treated with folate exhibited a proliferation of young leaves akin to the phenotype seen post-inoculation with the JQ_R2 strain (Fig. 3D). After drought stress, in contrast to the control group showcasing extensive leaf curling and wilting, the folate-treated *F. cymosum* plants demonstrated wilting primarily at the margins of some mature leaves, while the younger leaves displayed no evident symptoms (Fig. 5A). Upon quantifying the folate content in the leaves (Fig. 5B), our analysis revealed that foliar application of folate elevated the folate levels in the leaves under normal conditions. Following drought stress, a substantial reduction in folate content was noted in the control group, whereas in the folate-sprayed experimental group, a slight non-significant decrease was observed. In contrast, DHFR enzyme activity notably decreased in the experimental group before drought but exhibited a substantial increase post-drought stress (Fig. 5C). Simultaneously, we assessed the soluble sugar content and peroxidase (POD) enzyme activity in the leaves of both the experimental and control groups following drought stress (Fig. 5D). Both parameters exhibited significant increases in the folate-sprayed experimental group, with the POD enzyme activity being notably elevated, resembling the physiological response observed post-inoculation with the JQ_R2 strain mentioned previously (Fig. 3C, E).

In the aforementioned experimental findings, we noted that the DHFR enzyme activity in folate-treated plants decreased before drought stress, whereas the folate content increased. This outcome prompted us to explore the potential correlation between the *DHFR-TS* gene and folate content. Hence, we conducted transient transformation to enhance the expression of the *DHFR-TS* gene in *F. cymosum* leaves and subsequently monitored alterations in leaf phenotype, folate content, and DHFR enzyme activity. The outcomes indicated a notable increase of over a hundredfold in the expression level of the *DHFR-TS* gene in the leaves post-overexpression (Fig. 5F), with the leaves exhibiting enhanced drought tolerance. Following detachment for 5 hours, the leaves overexpressing *DHFR-TS* exhibited a markedly reduced water loss rate compared to the control group (Ev group, which expressed an empty vector) (Fig. 5E). Additionally, the *DHFR-TS* overexpressing leaves displayed elevated folate content and DHFR enzyme activity (Fig. 5G). These findings imply a positive correlation between the *DHFR-TS* gene and folate content in *F. cymosum*. Hence, we postulate that the JQ_R2 strain may elevate folate levels in *F. cymosum* plants by amplifying the expression of the *DHFR-TS* gene.

Prior studies have demonstrated that endophytic fungi are capable of directly secreting metabolites for the benefit of their hosts [47, 48]. This observation inspired us to investigate the possibility of JQ_R2 secreting folate, utilizing a portion for its requirements while supplying the remainder to *F. cymosum* to enhance its drought stress resilience. Consequently, we assessed the folate secretion and DHFR enzyme activity of the JQ_R2 strain on standard plates (0% PEG6000) and drought plates (containing 12% PEG6000). Observing the phenotype images of JQ_R2 cultivated on varying media (Fig. 5H), it is evident that the growth rate of JQ_R2 appears unaffected by drought; in fact, it demonstrates accelerated growth on the drought plates. Nevertheless, the strain's overall condition was impacted. The mycelium exhibited a dense and thick white appearance on regular plates, whereas it appeared gray, sparse, and thin on drought plates. Moreover, a notable secretion of folate was observed within the mycelium of JQ_R2. The folate content in the mycelium notably decreased on drought plates, while there was a significant increase in DHFR enzyme activity. This indicates that JQ_R2 can secrete folate under drought conditions to benefit *F. cymosum* directly and boost the host's *DHFR-TS* expression, consequently enhancing the plant's folate secretion.

Discussion

Endophytic fungi in *F. cymosum* display significant diversity.

Endophytic fungi within plants constitute a remarkable reservoir of diversity, extensively present in diverse plant tissues such as roots, stems, leaves, and fruits [49]. These fungi establish intimate associations with their surrounding environment, notably the rhizosphere soil of their host plants. High-throughput sequencing has gained extensive utilization in microbial diversity studies compared to traditional microbial isolation and purification methods, owing to its superior sensitivity, resolution, and efficiency [50]. High-throughput sequencing technology revealed notable variations in the endophytic fungi inhabiting tea plants across diverse ecological niches and age groups [51]. Samples of *Sophora alopecuroides* collected from diverse habitats exhibited differing richness and diversity of endophytic

fungi [52], underscoring the significant impact of the native habitat on the distribution of endophytic fungi. This finding highlights the varying fungal taxa present in different plant tissues and implies a probable correlation between the distinctiveness of root endophytic fungi and the soil diversity in their environments. The study utilized MISeq high-throughput sequencing technology to analyze the ITS rDNA genes of 18 samples of *F. cymosum* collected from six native habitats in southwestern China. This thorough analysis aimed to assess the richness and diversity of endophytic fungi in various habitats and tissue parts of *F. cymosum*.

Notably, the HL site showed the highest number of OTUs (Fig. 1B), with the root samples (HL_R) demonstrating the highest Chao1 and Observed_species indices (Supplementary Table S6). Conversely, the Chao1 and Observed_species indices exhibited notably low values in the leaf tissues as opposed to the leaf samples from alternative sites. Specifically, the diversity of endophytic fungi in the roots of *F. cymosum* at the HL site was remarkably high, contrasting with the low diversity observed in the leaves. This suggests that the soil characteristics at the HL site likely significantly impact this variation. Microorganisms are abundant in soil, and plant roots can attract these microorganisms, such as fungi, from the rhizosphere soil [53]. The higher the number of microorganisms in the soil, the greater the likelihood of their colonization of the plant. The abundance of soil microorganisms is intricately linked to soil characteristics, where appropriate pH, moisture, temperature, and a nutrient-rich environment with minerals and trace elements are vital for their survival [54]. HL is situated in the southern region of Sichuan Province, characterized by ample rainfall, dense vegetation, and favorable temperatures that contribute to fertile soil, nurturing local plant life and serving as a favorable environment for microorganisms. This premise effectively accounts for the notably high richness of endophytic fungi observed in the roots of *F. cymosum* at the HL site. Nevertheless, this raises additional queries regarding the comparatively low fungal richness in the leaves. Our findings revealed that the leaves of *F. cymosum* at the LZ site displayed the greatest richness, with the NH site following closely behind. Analyzing data from all six native habitat sites (Supplementary Table S1, S7), we noted that the LZ and NH locations are at the highest elevations, experiencing minimal annual precipitation and the lowest precipitation levels during the driest quarter and month. Could these attributes correlate with the abundance of endophytic fungi? Extensive research indicates that certain endophytic fungi can aid their hosts in combating drought stress [17]. In drought stress, plant leaf tissues are typically the initial areas impacted [55]. Consequently, a greater diversity of endophytic fungi in the leaves could potentially contribute to aiding the host in drought resistance [56]. This becomes especially pertinent in elevated regions where intense ultraviolet radiation risks harming chlorophyll and other photosynthetic pigments in plants. Endophytic fungi can aid the host in improving photosynthesis and fostering plant growth [57]. Hence, it is logical to note the elevated richness of endophytic fungi in the leaves of *F. cymosum* at high-altitude, low-precipitation sites like LZ and NH.

Regarding various tissue segments, the stems exhibited the highest richness and diversity of endophytic fungi (Fig. 1F, G), aligning with the results presented by Li et al. and Shen et al. [49, 58]. When evaluating the attributes of distinct plant tissues, stems typically exhibit greater stability than roots and leaves, experiencing fewer influences from external environmental factors. Furthermore, stems play a crucial

role as conduits for water and nutrient transportation [59], offering abundant resources conducive to fungal proliferation. Furthermore, soil-based fungi may infect plants upward through the roots [53], whereas fungi entering through leaf stomata can trigger infections downward [60]. The stable environmental conditions, nutrient-rich resources, and diverse avenues for fungal contamination collectively contribute to the stems' peak diversity and richness of endophytic fungi. In essence, *F. cymosum* harbors a wide array and profusion of endophytic fungi, with variations dependent on the habitat and specific plant tissue types.

Endophytic fungi of the genera *Fusarium*, *Colletotrichum*, and *Ceratobasidium* in *F. cymosum* have the potential for drought resistance.

Much research is available on fungi categorized under *Fusarium*, *Colletotrichum*, and *Ceratobasidium*. Nonetheless, the predominant focus of researchers concerning fungi within these three genera has centered primarily on their pathogenic properties, particularly those within the first two genera [61–63]. In this study, we propose a novel perspective on the function of endophytic fungi within their host. Through the investigation of the link between endophytic fungi and the precipitation levels of their indigenous habitats, our analysis revealed a negative correlation between endophytic fungi of the genera *Fusarium*, *Colletotrichum*, and *Ceratobasidium* and the annual precipitation, as well as the precipitation during the driest quarter and month (Fig. 2B). This indicates a greater prevalence of these fungi in areas with reduced precipitation. Consequently, we hypothesize that *Fusarium*, *Colletotrichum*, and *Ceratobasidium* species within *F. cymosum* can endure drought conditions and aid the host in withstanding external drought stress. Despite the prevalent focus on the pathogenic nature of these three fungal genera, there is evidence advocating for their potential contributions to growth promotion and drought resilience.

The genus *Fusarium* encompasses a vast array of fungi, comprising over 200 identified species, and is extensively present in soil, plants, and aquatic ecosystems. Among the *Fusarium* species, *Fusarium oxysporum* and *Fusarium graminearum* stand out as notable entities, renowned for their pathogenic effects that trigger a range of wilt and scab diseases in crops such as tomato wilt, cotton wilt, wheat fusarium head blight, and corn fusarium head blight [64, 65]. Researchers have undertaken comprehensive investigations into the management of pathogenic fungi through various approaches, including biological control utilizing antagonistic microorganisms, the formulation of chemical fungicides, and the screening and cultivation of resistant crop varieties [66–68]. While certain strains of *Fusarium* fungi have attracted scrutiny because of their potent pathogenic characteristics, this does not encapsulate the entirety of the *Fusarium* species. Notably, *Fusarium equiseti* has been found to stimulate the growth of tobacco by augmenting stem length and biomass, thereby bolstering the plant's resilience to water deficit stress [69]. In a similar vein, Hatamzadeh et al. observed that *Fusarium avenacearum* not only does not exhibit pathogenic traits but also enhances corn germination, suggesting its potential as a biofertilizer [70].

Moreover, *Fusarium petersiae* displays a notable concentration of siderophore units and facilitates the growth of wheat [71]. Fungi belonging to the *Fusarium* genus can bolster rice's resistance to salt and drought stress by releasing hormones like gibberellins, auxins, and abscisic acid [72, 73]. Moreover, these fungi can enhance plant drought tolerance by stimulating the activation of host genes associated with drought resistance. For instance, the association with *Fusarium solani* triggers the enhancement of the gene responsible for thaumatin-like protein (ThAQGP) in tobacco, contributing to its development in dry conditions [74]. In our research, we discovered that *Fusarium sp.* (JQ_R2), retrieved from the roots of *F. cymosum*, exhibits elevated auxin secretion levels in vitro and demonstrates average growth on drought stress plates (Supplementary Fig. S4, S5). During drought stress, *Fusarium* fungi primarily inhabit the subterranean sections of plants [75], aligning with our fungal isolation trials where we isolated 10 *Fusarium* strains from the roots of *F. cymosum* (Supplementary Table S8). In follow-up plant inoculation assessments, *Fusarium sp.* displayed pronounced growth-enhancing and drought-resistant attributes (Fig. 3B-E). Hence, we postulate that the endophytic *Fusarium sp.* residing in the roots of *F. cymosum* potentially alleviates drought stress through the secretion of particular metabolites (like hormones, antioxidants, vitamins, etc.) or by stimulating heightened expression of drought-related genes in the host.

Colletotrichum's genus encompasses roughly 190 species [76], with many known for inflicting significant plant ailments. Referred to as "anthracnose diseases," the conditions induced by *Colletotrichum* species are recognizable by depressed necrotic tissue formations yielding clusters of orange conidia. Considerable research efforts have concentrated on unraveling the pathogenic mechanisms of anthracnose diseases and devising strategies for their control [61]. Nevertheless, a few researchers have explored novel applications for *Colletotrichum* fungi. Beyond generating toxins that provoke plant afflictions, like the phytotoxic colletotrichins A-C [77], *Colletotrichum* fungi can excrete diverse secondary metabolites that foster plant growth and maturation. These secondary metabolites comprise alkaloids, phenolics, and flavonoids alongside diverse enzymes that facilitate nutrient assimilation by the host, like cellulases and amylases [78].

Moreover, they synthesize hormone-like compounds, notably auxin, as a prime example [79]. Notably, in scenarios of phosphate deficiency, *Colletotrichum* species have demonstrated substantial enhancement of *Arabidopsis thaliana* growth [80], a beneficial impact mediated by the serine biosynthesis phosphorylated pathway [81]. *Ceratobasidium* fungi frequently act as symbiotic partners in *Orchidaceae* orchids, furnishing orchid seeds with essential carbon and mineral nutrients, thereby fostering the germination, growth, and maturation of diverse orchid seeds [82]. Fungi belonging to the *Ceratobasidium* genus are renowned for their advantageous symbiotic roles. Besides forming associations with orchids, *Ceratobasidium* fungi interact with apple trees, stimulating growth through auxin secretion and fostering a mutualistic bond [83]. The relationships between endophytic fungi and plants are intricate and multifaceted. Not all fungal species exhibit strong pathogenicity within the genera *Fusarium* and *Colletotrichum*. Although we acknowledge that fungi of the same genus may possess shared characteristics, we propose that not all of them necessarily produce potent toxins. However, the quantity of toxins secreted can vary considerably, influencing differences in their pathogenic nature. Subtoxic

toxins, inadequate to induce disease, can paradoxically stimulate plant growth and bolster the plant's resilience against biotic and abiotic pressures [84]. These mild "pathogens" operate like vaccines, serving as growth stimulants and actively engaging in the plant's growth and maturation progression [85].

Exploring the correlation between folate, the JQ_R2 strain, and drought resistance in *F. cymosum*

Folate is vital in nucleic acid synthesis across plants, animals, and microorganisms. It is involved in amino acid and one-carbon metabolism and is closely linked to cell division and immune regulation [20]. Folate plays essential roles in cellular metabolism, growth, and development, rendering it a crucial nutrient for organismal survival. The activity of folate hinges on dihydrofolate reductase (DHFR), which converts dihydrofolate into tetrahydrofolate, the active form directly engaged in DNA synthesis, amino acid metabolism, and one-carbon unit metabolism [86]. Dihydrofolate reductase-thymidylate synthase (DHFR-TS) is a dual-function enzyme. In addition to its DHFR activity, the TS segment facilitates the conversion of deoxyuridylate (dUMP) to deoxy thymidylate (dTMP), a pivotal stage in DNA synthesis [87]. In this investigation, a comprehensive analysis of the transcriptome and metabolites revealed that under drought stress, the folate levels in *F. cymosum* rose following inoculation with the JQ_R2 strain, along with an upsurge in the expression of the *DHFR-TS* gene (Fig. 4H).

Moreover, quantitative real-time polymerase chain reaction (qRT-PCR) validation affirmed that the expression levels of essential genes in the folate synthesis pathway were heightened post-inoculation with the JQ_R2 strain (Fig. 4I). These findings prompt us to hypothesize that the JQ_R2 strain aids *F. cymosum* in combating drought stress through the folate metabolism pathway. Consequently, we conducted foliar folate spraying to ascertain if folate could enhance *F. cymosum*'s drought resistance, with positive outcomes (Fig. 5A). Furthermore, there was an elevation in folate content in the leaves, accompanied by increased DHFR enzyme activity (Fig. 5B, C). Aside from *F. cymosum*, external administration of folate has been shown to improve the resistance of *Alternaria brassicicola*, wheat, *Phaseolus vulgaris* L., and *Coriandrum sativum* L., encompassing resistance against diseases, salinity, and drought[21, 37, 88]. Moreover, transiently elevating the expression of *DHFR-TS* resulted in amplified folate content and DHFR enzyme activity in the leaves, consequently augmenting the drought resistance of *F. cymosum* leaves (Fig. 5E-G). Furthermore, we validated that the JQ_R2 strain secretes folate under both normal and drought conditions, with a notable rise in DHFR enzyme activity observed under drought conditions (Fig. 5H). Following the outcomes above, we infer that the JQ_R2 strain aids *F. cymosum* in counteracting drought stress by elevating folate content and DHFR enzyme activity. This circumstance is not singular; akin results have been documented in strawberries and *Moringa oleifera*, where inoculation with growth-promoting strains led to enhanced folate content and DHFR enzyme activity in the host, subsequently fostering host growth and development [22, 89].

Moreover, humans cannot synthesize *folate de novo* and thus must acquire it from vegetables. Folate deficiency is widespread, resulting in congenital disabilities and anemia [90]. Various endeavors have been undertaken to boost folate levels in food through biofortification, with fungal fermentation as a viable approach [91]. In this study, the inoculation of *F. cymosum* with the JQ_R2 strain raised folate

levels during drought conditions. This elevation strengthened the plant's resilience to external stress and enhanced its nutritional value as a vegetable fit for consumption.

Conclusions

Plant growth-promoting endophytic fungi are promising and environmentally friendly biofertilizers that can stimulate plant growth and improve plant resilience. In this study, we introduced a novel methodology that links endophytic fungi to the precipitation levels of their natural environments to pinpoint drought-resistant endophytic strains. Our investigation verified that three chosen strains exhibit drought-resistant properties with growth-promoting capabilities and demonstrated that JQ_R2 enhances the drought resilience of *F. cymosum* by boosting folate content and DHFR enzyme activity. These discoveries enhance our comprehension of how endophytic fungi boost host resilience and reinforce our assurance in transforming beneficial endophytic fungi into biofertilizers. Such progress opens new avenues for research to enhance plant adaptation to severe drought conditions.

Declarations

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Authors' contributions

MQC: Conceptualization and Writing original draft. ZQD: Conceptualization and Statistical analysis. QFL, TLB and ZZT: Materials and Methodology. CLL, QW and JZW: Materials and Sampling. KXZ: Conceptualization, Statistical analysis, and Data visualization. MLZ and HC: Acquisition and Project administration.

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Availability of data and materials

The ITS high-throughput sequencing data and transcriptome sequencing data from this study are available at NCBI under BioProject ID PRJNA1135529 and PRJNA1135705, respectively. The metabolomics data can be accessed in Metabolights using the identifier MTBLS10685. Additional figures and tables not presented in the article can be found in the supplementary materials.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

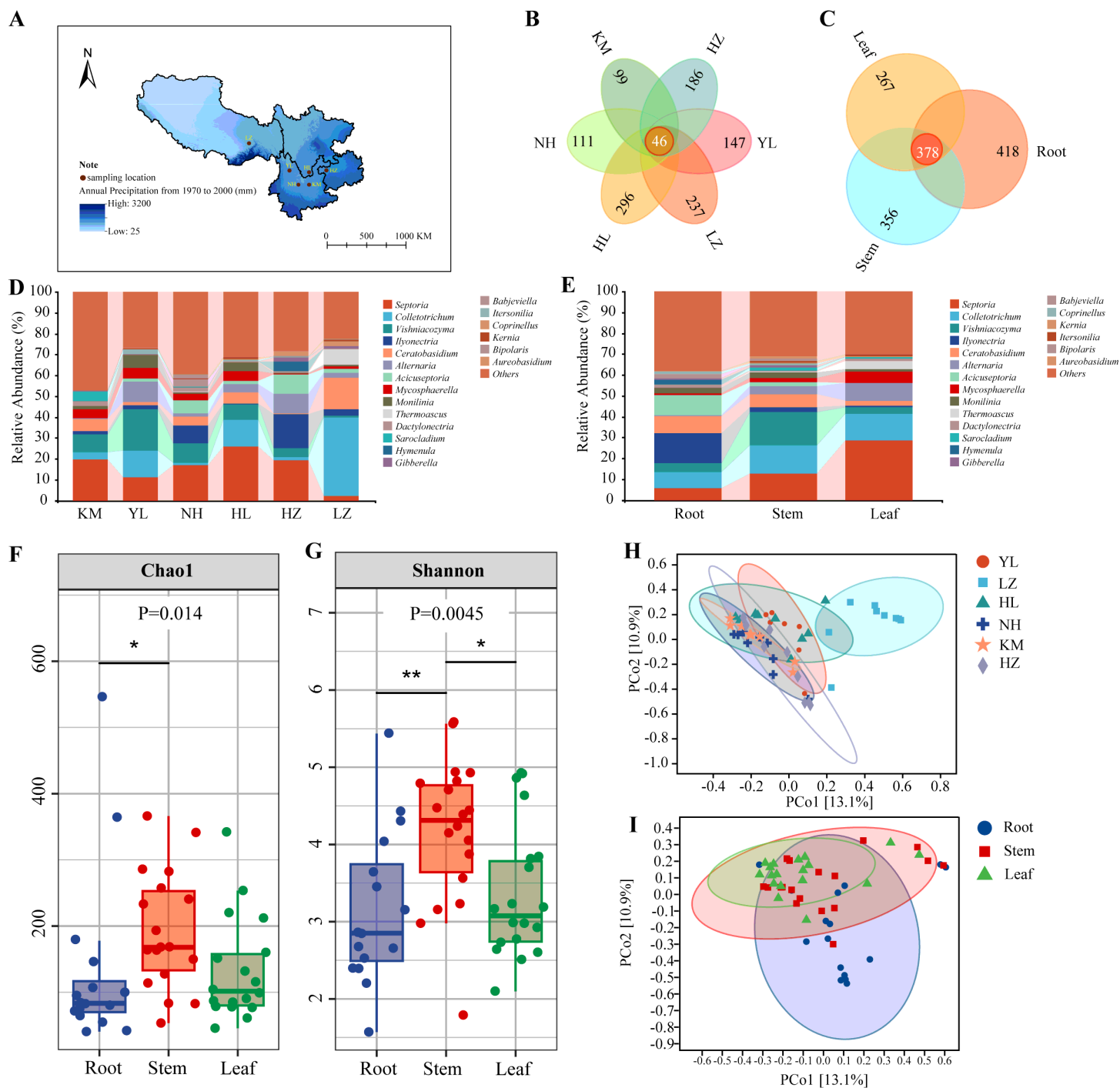


Figure 1

Diversity of endophytic fungi in *F. cymosum* from various habitats and tissue sites. **(A)** Displays maps indicating the habitat status of six wild *F. cymosum* locations. A blue gradient scale ranging from light to dark signifies the annual precipitation levels from low to high. Abbreviations representing the site names are designated by letters in the figure (LZ: Linzhi County, Tibet Autonomous Region; YL: Yulong County, Yunnan Province; NH: Nanhua County, Yunnan Province; KM: Kunming City, Yunnan Province; HL: Huili County, Sichuan Province; HZ: Hezhang County, Guizhou Province). **(B, C)** Venn diagrams depicting the genus-level endophytic fungi distribution in varying habitats and tissue sites (roots, stems, and leaves) of

F. cymosum. (D, E) Histograms displaying the genus-level endophytic fungal composition of *F. cymosum* across various habitats and tissue sites reveal the top 20 species abundances at the genus level. (F, G) Box plots illustrating Alpha diversity across distinct tissue sites of *F. cymosum*. The Chao1 index reflects the richness of endophytic fungal communities, while the Shannon index represents the community's diversity. Asterisks denote significant group disparities (*: $p < 0.05$; **: $p < 0.01$, ANOVA, Duncan's Multiple Range Test). (H, I) Principal Coordinate Analysis (PCoA), utilizing Weighted-UniFrac distance, revealed distinctions in endophytic fungal community composition among various habitats and tissue sites. Each sample is differentiated by a unique combination of colors and shapes for representation.

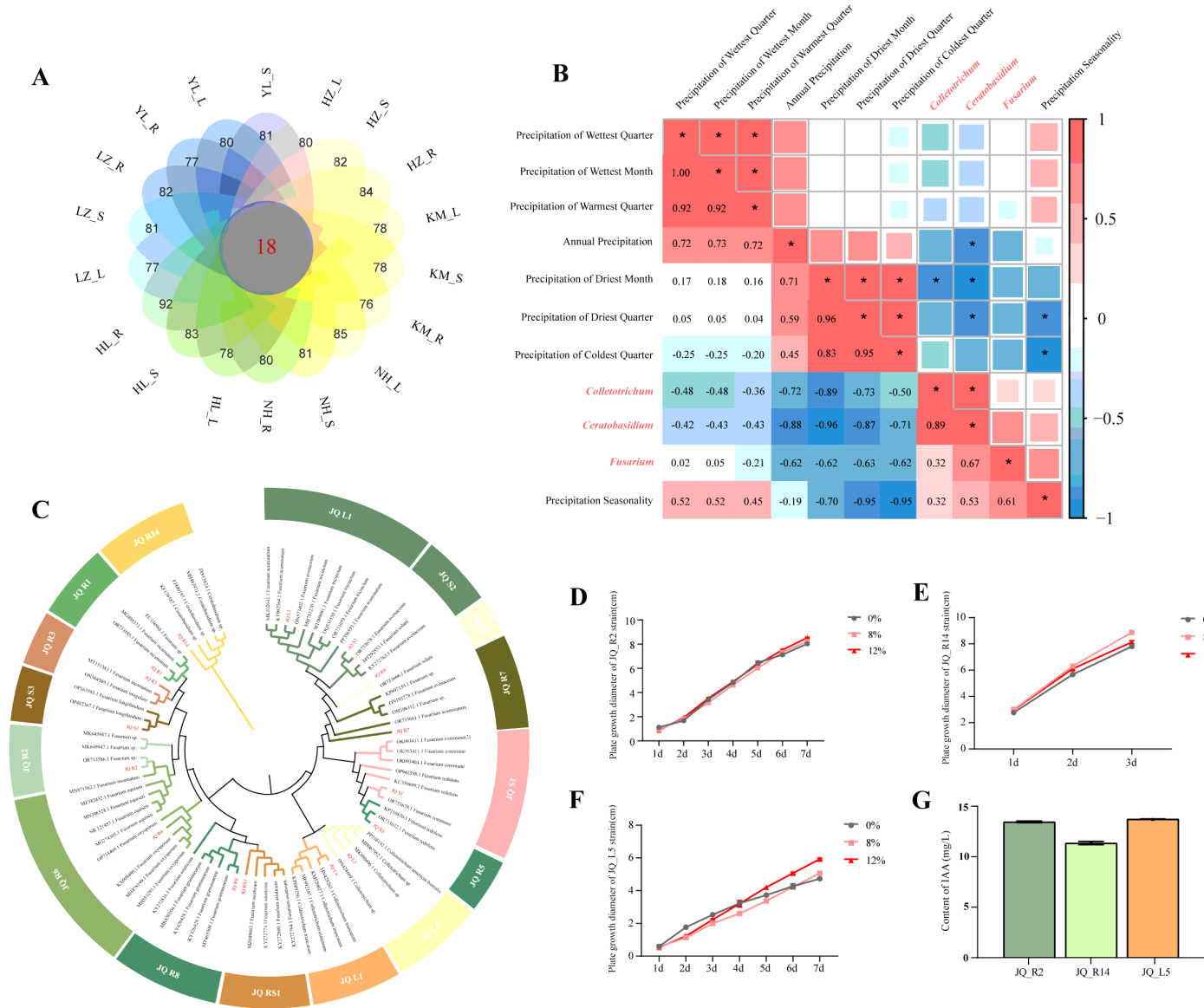


Figure 2

Endophytic fungi of the genera *Fusarium*, *Colletotrichum*, and *Ceratobasidium* inhabiting *F. cymosum* exhibit a negative correlation with precipitation factors. Furthermore, specific strains have demonstrated the ability to thrive under drought conditions and secrete auxins in vitro. (A) Common genus-level endophytic fungi within 18 sample groups representing six diverse habitats and three distinct tissue

sites. The abbreviations are R for roots, S for stems, and L for leaves. **(B)** Correlation analysis between endophytic fungi at the genus level, specifically *Fusarium*, *Colletotrichum*, and *Ceratobasidium*, and eight environmental factors related to precipitation using Pearson correlation analysis. Red and blue colors indicate positive and negative correlations, respectively, and * indicates significant correlation. **(C)** Phylogenetic trees, constructed using the neighbor-joining method, depict the relationships among endophytic fungi within the 16 target genera. **(D, E, F)** JQ_R2, JQ_R14, and JQ_L5 growth was observed on drought-selective medium. Grey lines represent standard medium; light red lines indicate plates with 8% PEG6000 supplementation for drought conditions, while red lines indicate plates supplemented with 12% PEG6000 for severe drought conditions. **(G)** In vitro secretion of the growth hormone IAA by JQ_R2, JQ_R14, and JQ_L5.

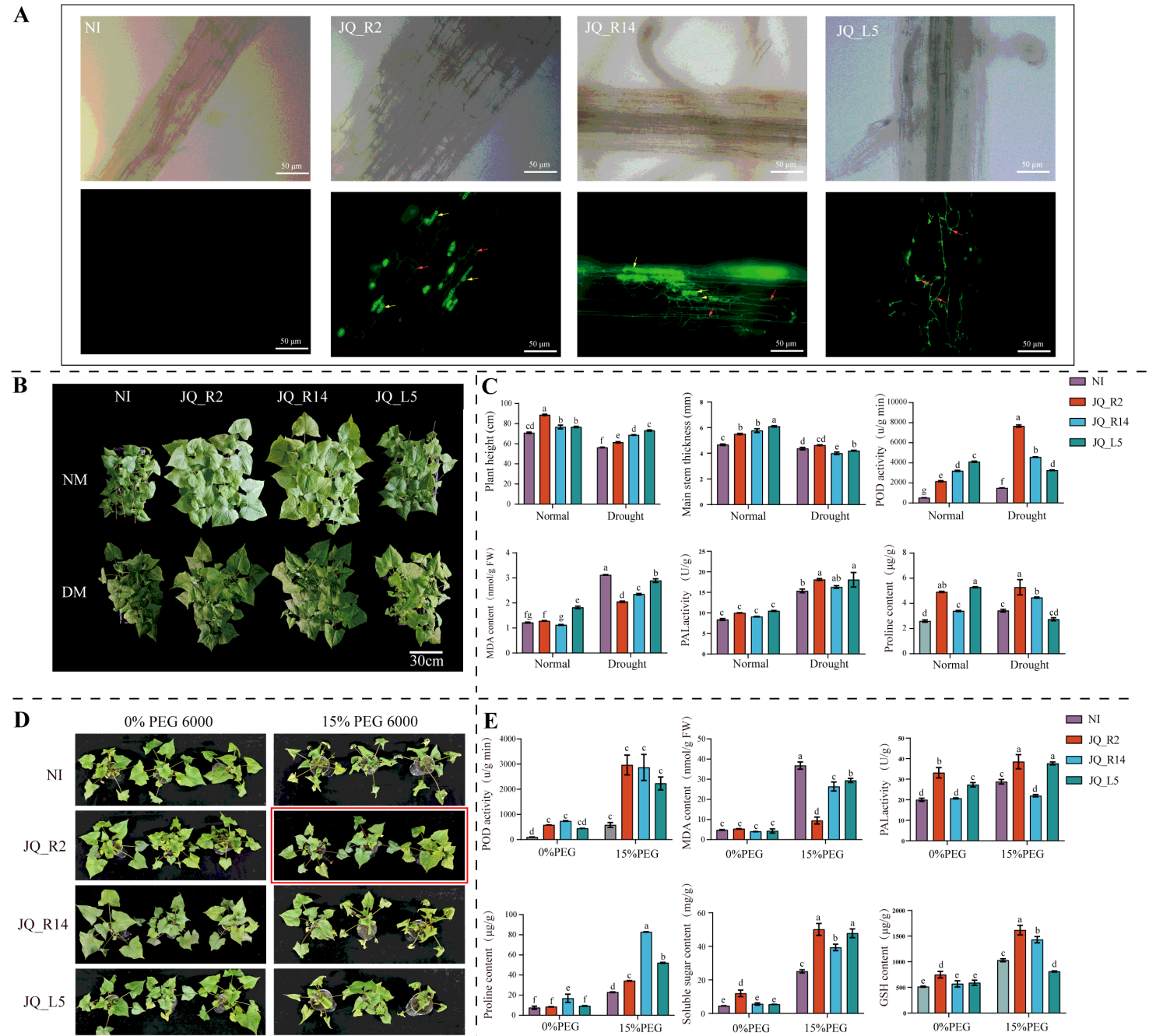


Figure 3

JQ_R2, JQ_R14, and JQ_L5 exhibit the ability to colonize the roots of *F. cymosum*, enhance the growth of *F. cymosum* seedlings cultivated in soil and hydroponic systems, and mitigate the impact of drought stress on the plants. **(A)** JQ_R2, JQ_R14, and JQ_L5 colonize the roots of *F. cymosum* plants, as shown through WGA-AF488 fluorescence staining. In the images provided, the upper and lower images correspond to the white-field and fluorescence-field photographs, respectively. The term "NI" denotes plants not inoculated with exogenous fungi. (Red arrows highlight mycelium, while yellow arrows indicate sclerotium). **(B, C)** The growth of soil-cultivated *F. cymosum* plants was evaluated under NM and DM, alongside variations in agronomic traits and physiological indicators. These assessments encompassed uninoculated (NI) plants and those inoculated with JQ_R2, JQ_R14, and JQ_L5, involving parameters such as plant height, central stem thickness, POD enzyme activity, MDA content, PAL activity, and proline content. **(D, E)** Hydroponic *F. cymosum* plants were subjected to a standard nutrient solution and a nutrient solution with 15% PEG6000 to replicate drought stress. The growth of the plants and alterations in various physiological indicators were then assessed in uninoculated (NI) plants and those inoculated with JQ_R2, JQ_R14, and JQ_L5. These assessments covered activities of POD and PAL enzymes, levels of MDA, proline, soluble sugars, and GSH. Significant differences are denoted by distinct letters, with a significance level set at $p < 0.05$.

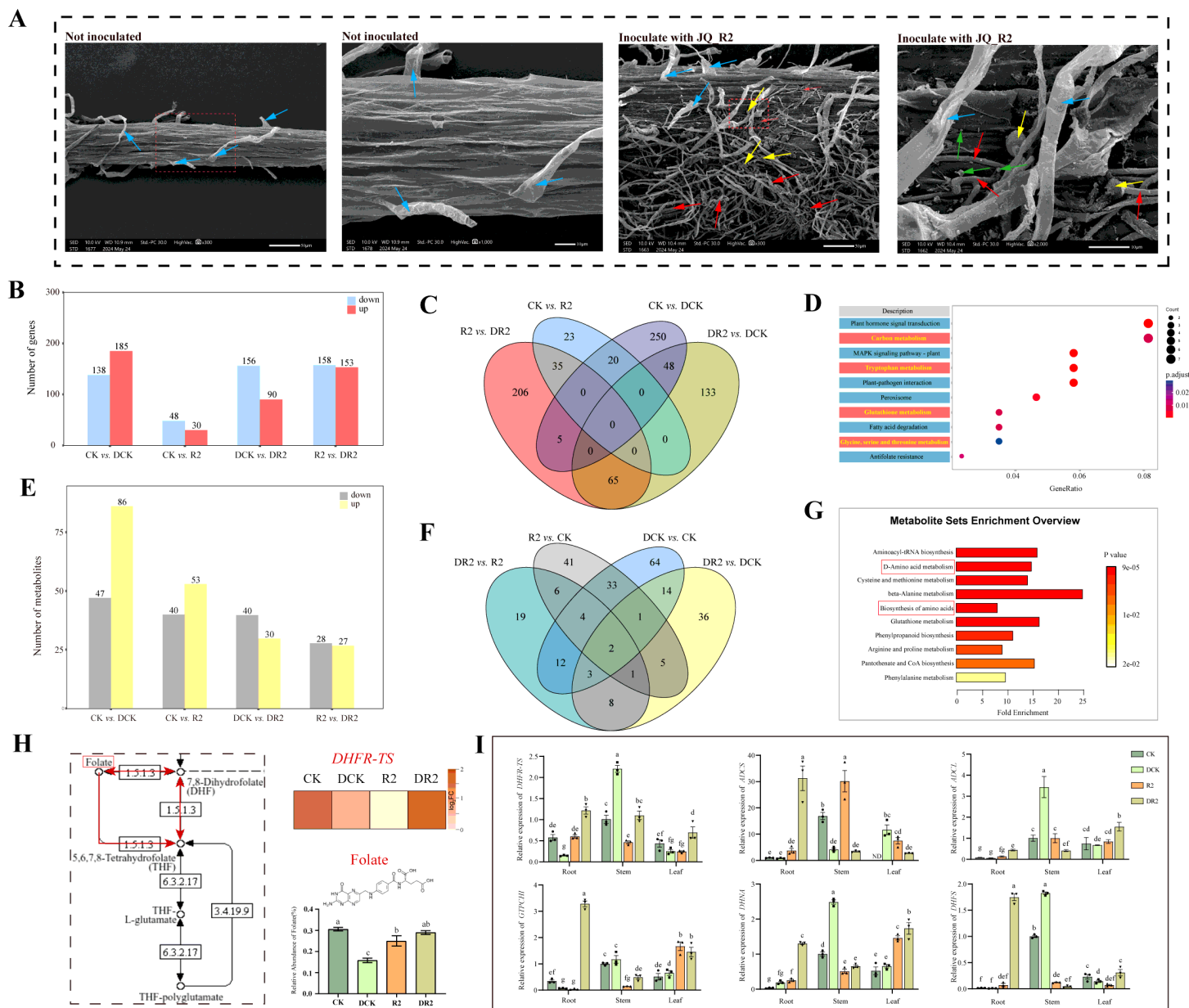


Figure 4

Multi-omics analysis of how JQ_R2 mitigates drought stress in *F. cymosum*. **(A)** Scanning electron microscopy images depict the surface of *F. cymosum* roots post JQ_R2 colonization (and non-colonized roots). Blue arrows indicate root hairs, red arrows highlight hyphae, yellow arrows denote sporangia, and green arrows indicate spores. **(B)** The bar chart illustrates the counts of differentially expressed genes (DEGs) in the four comparison groups: CK for average moisture without fungal inoculation, DCK for drought conditions without fungal inoculation, DR2 for drought conditions with JQ_R2 inoculation, and R2 for average moisture with JQ_R2 inoculation. **(C)** Venn diagram of DEGs. **(D)** KEGG enrichment pathways analysis of DEGs in the DR2 versus DCK comparison. **(E)** The bar chart displays the differentially expressed metabolites (DEMs) count in the four comparison groups. **(F)** Venn diagram of DAMs. **(G)** Analysis of KEGG enrichment pathways for DAMs in comparing DR2 and DCK. **(H)** Based on KEGG enrichment analysis, genes and metabolites involved in carbon metabolism, amino acid

metabolism, and biosynthesis exhibited enrichment in both the transcriptome and metabolome. Notably, there was a significant upregulation in the expression of folate and Dihydrofolate Reductase-Thymidylate Synthase (DHFR-TS) in the DR2 experimental group compared to the DCK group. (I)Real-time quantitative PCR was utilized to validate the expression of crucial genes implicated in folate synthesis across the four experimental groups and various tissue samples. The data are presented as mean $\pm$ standard deviation, n = 3 (where different lowercase letters signify significant variations, $p < 0.05$).

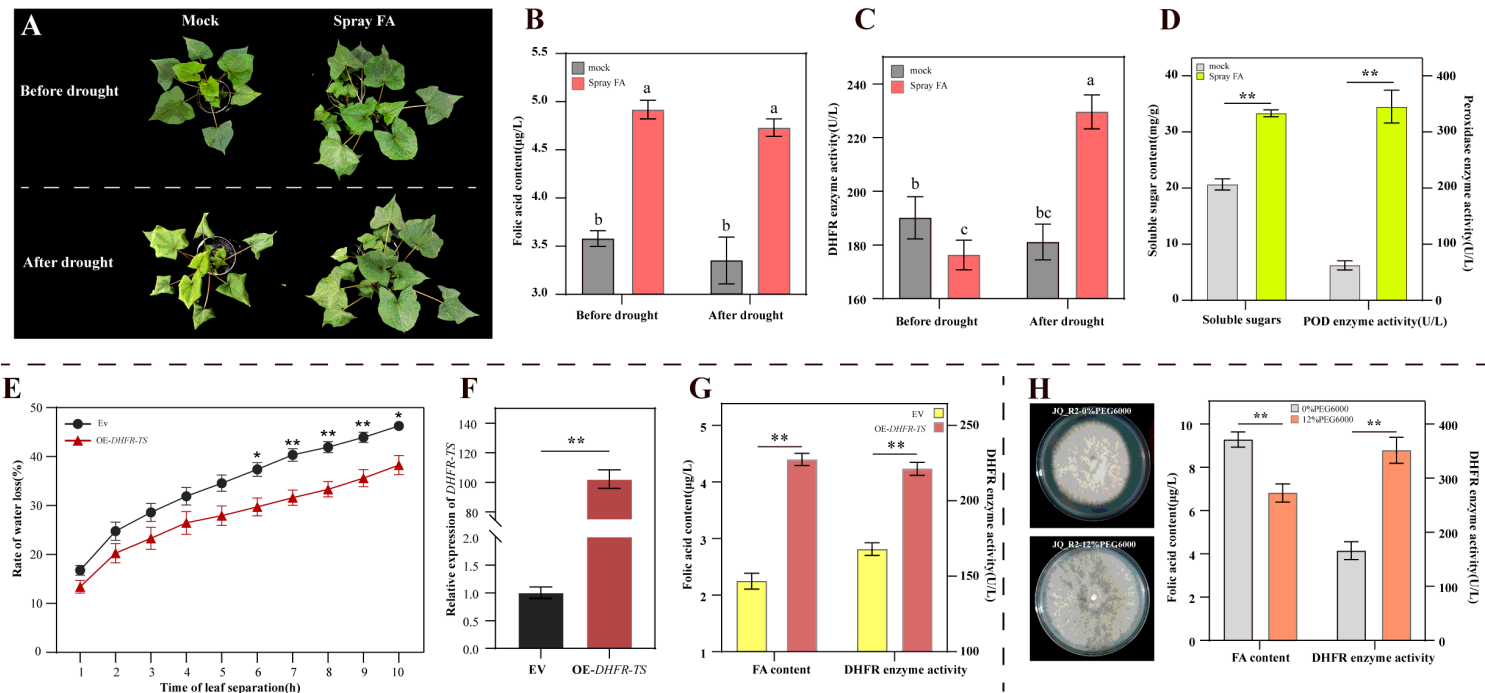


Figure 5

Folate can help *F. cymosum* plants resist drought stress. (A, B, C) The phenotypic comparison was conducted on *F. cymosum* plants cultivated hydroponically. The comparison involved plants before and after experiencing drought stress, followed by spraying with folate (Spray FA) or distilled water (mock). Additionally, the investigation included the analysis of folate content and DHFR enzyme activity in the leaves. (D)Following the drought stress, the alterations in the soluble sugar content and peroxidase (POD) enzyme activity in the leaves of *F. cymosum* were quantified. (E, F, G) transient overexpression of *DHFR-TS* (Ev serving as the control empty vector), various parameters were assessed in *F. cymosum* leaves, including the rate of detached leaf water loss, the relative expression level of the *DHFR-TS* gene, folate content, and DHFR enzyme activity. (H) Morphological images of the JR_R2 strain cultivated on PDA plates containing 0% and 12% PEG6000 for 5 days and measurements of folate content and DHFR enzyme activity in the mycelium were captured. The data are presented as mean $\pm$ standard deviation, n = 3, where distinct lowercase letters denote statistically significant variances ($p < 0.05$), and asterisks (*) highlight significant differences between the data (* denotes $p < 0.05$, ** denotes $p < 0.01$).

Supplementary Files

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