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ORIGINAL PAPER

Plant sexual variation modulates rhizospheric nutrient processes through the soil microbiome response to drought and rewetting in *Populus cathayana*

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Abstract
Drought affects the physiological environment of the soil microbiome and plants. However, the ability of drought and rewetting to regulate sex-specific soil nutrient availability with respect to bacterial- and fungal-mediated rhizospheric processes has not been clarified. The impact of drought and rewetting on soil bacterial and fungal communities and rhizospheric processes were examined in dioecious *Populus cathayana* females and males. Drought and rewetting affected the composition and abundance of the bacterial and fungal communities but not their diversity in the rhizosphere of both sexes. Male had more drought-tolerant fungi and bacteria in the rhizosphere of males than females in response to drought. The changing bacterial and fungal community composition promoted soil ammonification in the rhizosphere of female plants, whereas nitrification by ammonia-oxidizing archaea (*AOA*) and ammonia-oxidizing bacteria (*AOB*) was accelerated in the rhizosphere of male plants. Rewetting reduced the sexual differences in the ratio of nitrate (NO_3^-) to ammonium (NH_4^+) in the rhizosphere. In the rhizosphere of females, the changes in fungal community composition were linked more strongly to soil N and P reactions during recovery compared with that in the bacterial community composition. Conversely, the bacterial community rather than fungal components was the main contributor to soil N reactions in the rhizosphere of males. Plant sex influenced the response and recovery of fungal and bacterial communities to drought, and sexual dimorphism was detected in the regulation of rhizospheric nutrient reactions. Our results emphasize the ecological relevance of plant sex in determining the response of soil microbial communities to drought and rewetting effects and their ecological functions in *P. cathayana*.

Keywords Dioecy · Enzyme activities · Microbial diversity · Rewetting

Introduction

Drought affects tree growth and population distribution not only during the drought period but also afterwards. Delayed effects on vegetation may last for years despite the alleviation of drought (Kannenberg and Phillips 2020). Females and males of diecious tree species exhibit sexual dimorphism, including not only the sexual organs but also secondary sexual traits, such as morphology, physiology, and nutrient availability traits (Thomas and LaFrankie 1993; Juvany and Munné-Bosch 2015). Sexual differences in functional traits of dioecious plant species would be greater in response to drought stress. Some dioecious species may be especially vulnerable to the effects of drought, and recovery could be difficult even after rewetting because they often display spatial segregation of sexes (Hultine et al. 2016). Therefore, it is important to understand the response of dioecious tree species to drought and rewetting.

Drought can change the composition and activity of soil microbial communities (Fierer et al. 2003; Leizeaga et al. 2022) and influence processes related to nutrient cycling and primary production (Najera et al. 2020). Soil bacterial and fungal communities respond differently to drought depending on their differential tolerance and metabolic flexibility (Allison and Martiny 2008). Generally, bacterial communities are more sensitive and less resistant to drought than are fungal communities (de Vries et al. 2018). The differential responses and tolerance of fungal and bacterial communities

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to drought probably underlie the difference in their abilities to recover from drought. Generally, fast-responding microbes occupy niches earlier than slow-responding species upon rewetting; this may be closely related to soil function during recovery from drought (Meisner et al. 2021). However, other studies have found that some fungal species are more sensitive or opportunistic to drought than bacteria (Crowther et al. 2014; Meisner et al. 2018). Various microbial taxa possess different degrees of tolerance and responses to soil water availability (Evans et al. 2020). Thus, relative to individual microbial strains, the broader contribution of networks of co-existing soil microbiomes is more important for predicting microbial responses to drought and subsequent rewetting and thus for predicting soil functioning. For example, De Vries et al. (2018) showed that soil bacterial networks were less stable than fungal networks under drought conditions, and the linkage of bacterial communities with soil function was stronger during recovery from drought than that of fungal communities. However, little is known about how bacteria and fungi respond to and recover from drought upon rewetting or about the linkage of microbial communities' properties with soil function in dioecious plant species.

Much recent effort has been made to explore the use of rhizospheric microbial communities to alleviate plant drought stress (de Vries et al. 2018). Changes in soil water affect plant growth and photosynthesis, which regulate carbon allocation below ground so that the C released is differently allocated into the soil microbial community (Fuchslueger et al. 2014). This could, in turn, select microbial populations (Jones et al. 2004; Berg and Smalla 2009) that enable plants to cope with water stress and recover from drought (Preece and Penuelas 2016). Soil microbes mineralize soil organic matter, improving soil structure and quality and providing sufficient soil nutrient content to support plant survival under drought conditions (Nannipieri et al. 2020; Canarini et al. 2021). Also, soil microbes produce various growth-promoting factors, such as hormones and enzymes (Gargallo-Garriga et al. 2018). The hormones, such as auxin, secreted by soil microbes, promote root elongation and thus the exploration for soil nutrients, thus increasing plant tolerance to drought stress (Raheem et al. 2018). Previous studies have suggested that the preference of plants for nutrient forms, such as NO_3^- or NH_4^+, affects plant performance in response to a stressful environment (Chen et al. 2013; Kronzucker et al. 1997). Females and males exhibit sexual dimorphism in leaf photosynthesis, nutrient absorption, C and N accumulation, allocation and metabolism, root traits, and biomass accumulation in response to drought stress (Liu et al. 2021b, 2022a). Such sexual dimorphism may have a cascade effect on C allocation into the roots and root exudate metabolites and thus on rhizospheric microbial community composition and function (Xia et al. 2021). Moreover, soil moisture has been shown to affect sex-specific N form preference, in that poplar females prefer to absorb NH_4^+, while males prefer to use NO_3^- from the soil under drought stress (Chen et al. 2013). However, it remains unclear whether the preferences of plants for nutrient levels or forms are affected by soil microbial community composition, nor do we know how sex differences affect the responses of rhizospheric microbial communities to changed soil water conditions.

In this study, we used *Populus cathayana* females and males to explore sex-related differences in their microbial communities and their responses to drought and subsequent rewetting, as well as their influence on soil function. We attempted to address the following questions: (i) Do sex-specific responses to drought and rewetting affect the properties of rhizospheric bacterial and fungal communities? (ii) How do the properties of bacterial and fungal communities regulate the legacy effects of drought and their ecological functions? Based on this, amplicon sequencing was performed on the rhizosphere-associated microbial community of poplar females and males to investigate the microbial responses to drought and rewetting and their ecological functions.

Materials and methods

Plant materials and experimental design

P. cathayana cuttings were collected from 32 different maternal trees (16 of each sex) from the habitats of Qinghai Province, China (30° 67′ N, 104° 06′ E 30° 67 N, 104° 06 E), as explained in detail by Liu et al. (2020). Cuttings (15–20 cm in length with 2–4 buds) were rooted in a glasshouse with natural light at the Hangzhou Normal University of Hangzhou, Zhejiang Province (China, 30.03° N, 120.120° E). After cultivation for 4 weeks, seedlings were transplanted into 10-l pots of 10 kg soil with 122.6 mg kg^{-1} available P, 1.8 g kg^{-1} total N (TN), 52.7 mg kg^{-1} NH_4^+-N, 476.5 mg kg^{-1} available K (AK), 33.3 g kg^{-1} soil organic matter content (OM), and 106.3 mg kg^{-1} NO_3^--N, for 6 weeks. Afterwards, the experiment was conducted with two sexes (female, F; male, M) and two water regimes (control CK1, drought D), with eight replicates per treatment. After cultivation for 12 weeks, four seedlings were harvested in each treatment. The remaining plants were re-watered to 100% field capacity for the next 6 weeks, which produced four treatments, sex (F and M) × water regime (control 2 (CK2) and rewetting after drought (DW)), with four replications per treatment. For CK2, the pots were weighed daily to maintain 100% field capacity. For DW, pots were weighed daily to maintain 30% field capacity (Fig. 1).

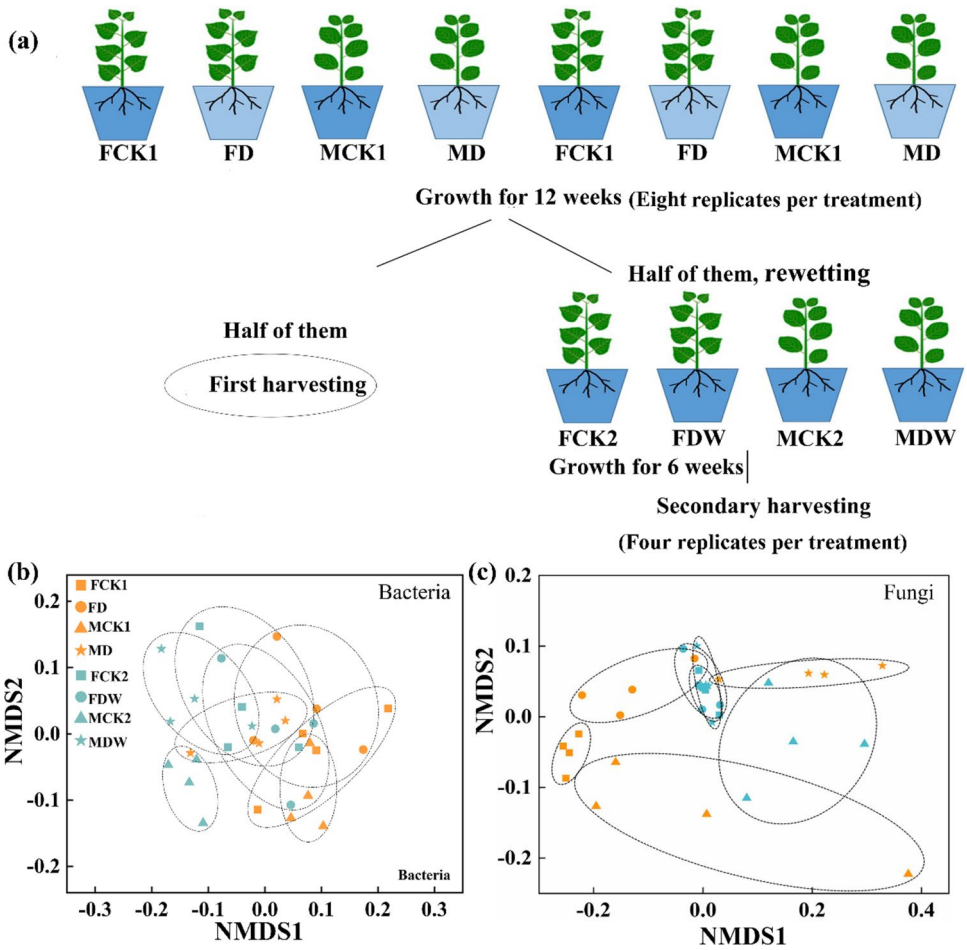

Fig. 1 Schematic diagram of experiment layout (**a**) used in this study and microbial beta diversity indices (**b**, **c**). The beta diversity indices show the nonmetric multidimensional scaling (NMDS) of bacterial and fungal communities. The experiment was conducted with two sexes (female, F; male, M) × two water regimes (control CK1, drought D), with eight replicates per treatment. After cultivation for 12 weeks, four seedlings were harvested in each treatment. The remaining plants were re-watered to 100% field capacity for the next 6 weeks, which produced four treatments, sex (F and M) × water regime (control 2 (CK2)) and rewetting after drought (DW) with four replications per treatment. For CK2, the pots were weighed daily to maintain 100% field capacity. For DW, pots were weighed daily to maintain 30% of field capacity. FCK1, female with previous control; FD, female with drought stress; MCK1, male with previous control; MD, male with drought; FCK2, female with control; FDW, female with rewetting; MCK2, male with control; MDW, male with rewetting

Plant harvesting and soil sampling

After 12 weeks of continuous drought, four seedlings from each treatment were harvested, and the roots were carefully removed from each pot. The shoots were separated into stems and leaves. The soil adhering to the roots was collected as rhizosphere soil by gently shaking the plant roots. Rhizosphere soil samples were divided into four parts: one part was air-dried at room temperature; one part was stored at 4 °C until measuring the soil enzyme activity, microbial C and N, and pH, which were performed within one week; one part was oven-dried at 105 °C to quantify the soil water content; and the remaining part was stored at −80 °C until DNA extraction. The remaining seedlings were watered well for 6 weeks and then harvested as described above.

Soil physicochemical properties

After sieving (~2 mm), the soil pH was measured with glass electrodes connected to a PHBJ-260 pH metre (Shanghai Leici, China) at a ratio of 1:2.5 soil to H_2O (Fu et al. 2016). The soil cation exchange capacity (CEC) was measured using the ammonium acetate method (Kitsopoulos 1999). Total N and C were measured using a chemiluminescence detector (CLD-TOC) coupled with a C and N analyser (multi C/N 3100; Jena Analytics, Jena, Germany). The soil total K content was determined by plasma-atomic emission spectrometry (ICPS-7500, Shimadzu, Japan) after digestion with H_2SO_4 and $HClO_4$ (Zhang and Gong 2012). Soil total phosphorus (TP) was determined using molybdenum blue colorimetry after digestion with $HClO_4$ and H_2SO_4 (Olsen

et al. 1954). After extraction with 1 M KCl, the NO_3^--N and NH_4^+-N contents of soil samples were measured using a continuous flow analytical system (SEAL Analytical Auto Analyzer 3) (Sahrawat and Prasad 1975; Sims and Jackson 1971). Fresh soil samples were used to measure the soil microbial N and C levels with chloroform fumigation after extraction with 0.5 M K_2SO_4 (1:5 ratio of soil to solution) (Brookes et al. 1985; Vance et al. 1987). The microbial C and N contents were defined as the difference in C and N contents between non-fumigated and fumigated samples, with an EN factor of 2.64 used for microbial C and an EC factor of 2.22 used for microbial N (Brookes et al. 1985; Joergensen et al. 1995). The content of soil OM was measured with an elemental analyser after removing inorganic C with 0.5 M HCl (Nelson and Sommers 1982). The net nitrification rate was determined from the changes in NO_3^- extracted using 2 M KCl after soil incubation in the dark at 20 °C for 14 days (Zhang et al. 2019). The net soil ammonification rate (AMR) was determined from the changes in NH_4^+ under the NNR measurement conditions. Each treatment was replicated four times.

Soil enzyme activities

Soil urease (URE) activity was measured colorimetrically at 690 nm by measuring the released NH_4^+ from the reaction solution of 5 g fresh soil sample with 2.5 ml of a 0.08-M urea solution for 2 h at 37 °C (Kandeler and Gerber 1988). The activity of nitrate reductase (NR) in soils was measured at 520 nm after incubation at 30 °C for 24 h (Nowak et al. 2002; Hu et al. 2018). Soil nitrite reductase (NIR) activity was determined by the reduction of nitrite to ammonium using a mixture of 50 mM $NaNO_2$ and 5 mM methyl viologen (Krishna et al. 1988). Protease activity (PRO) was determined as previously described by Ladd and Butler (1972). Briefly, 5 g of soil was mixed with 2.5 mL of Na-caseinate solution (2%) and 2.5 mL of 0.2 M Tris buffer (pH 8.0) and incubated for 2 h at 50 °C. The supernatant was mixed with 0.25 mL of Folin-Ciocalteu reagent and 0.75 mL of Na_2CO_3 (1.4 M) after precipitation with 5 mL of trichloroacetic acid (10%) and measured at 680 nm after 5 min. Polyphenol oxidase (PPO) activity was assayed using 1 g of fresh soil with 4 mL of citric acid phosphate (buffered to pH 4.5) and 10 mL of 1% pyrogallic acid incubated at 30 °C for 2 h (Yang et al. 2017). CAT activity was determined by treating a mixture of 5 g soil, 5 mL H_2O_2 (0.3%), and 5 mL sulfuric acid (1.5 mol L^{-1}) with $KMnO_4$ (Wu et al. 2016) and performing measurements after the soil samples were inoculated at 25 °C for 20 min in the dark. Soil L-glutaminase (GLS) activity was measured as described by Frankenberger and Tabatabai (1991). β-glucosidase (GU) activity was assayed according to the method described by Tabatabai (1994). Briefly, 1 g of fresh soil was treated with a mixture containing 0.1 M trihydroxymethyl aminomethane, 0.067 M citric acid monohydrate compound, and 0.1 M boric acid followed by the addition of 0.05 M p-nitrophenyl-β-D-glucopyranoside. After incubating for 1 h, the soil mixture was treated with 0.1 M trihydroxymethyl aminomethane and 0.5 M $CaCl_2$ and the optical density of the supernatant measured at 410 nm. N-acetyl-β-D-glucosaminidase (NAG) activity was determined based on a method similar to that for GU, except p-nitrophenyl-β-d-glucopyranoside was replaced with p-nitrophenyl-N-acetyl-β-d-glucosaminidine and pH 6.0 was used instead of pH 5.5 (Wang et al. 2015). Denitrification enzyme (DEA) activity was treated with 1 mM glucose, 1 mM KNO_3, and 1 g L^{-1} chloramphenicol and measured as described by Barnard et al. (2004). The produced N_2O was analysed using a gas chromatograph equipped with an electron capture detector (Varian Chromatography Group, CA, USA). Each treatment was replicated four times.

Sequencing processing and bioinformatic analysis

Soil samples (500 mg) were extracted using a PowerMax Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). After evaluating the quality of DNA, the V3-V4 hypervariable regions of the 16S ribosomal RNA were amplified for bacteria (338F: 5′-ACTCCTACGGGAGGC AGCAG-3′, 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) (Mori et al. 2014), and the internal transcribed spacer (ITS) (ITS3F: 5′-GCATCGATGAAGAACGCAGC-3′, ITS4R: TCCTCCGCTTATTGATATGC) (White et al. 1990) was amplified for fungi, by real-time PCR in a 20-μL reaction. A two-step PCR was performed. Amplicon primers and barcode index primers were ordered from Invitrogen (Carlsbad, CA, USA). During the first step, the sequencing adapters and Illumina-specific overhang adapter sequences for compatibility with the Illumina index were added. The PCR amplification was performed in 20 μL reactive solutions under the following conditions: 30 cycles of 95 °C for 30 s, 55 °C for 45 s, 72 °C for 60 s, and 72 °C for 10 min. The primers with unique index barcodes for each sample were added during the second step of the PCR, according to the above amplification conditions. The PCR products were pooled and purified using an Axyprep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. After purification, PCR products were quantified using a NanoDrop spectrophotometer (NanoDrop 2000C, Thermo Scientific).

After purification with an Agencourt AMPure Xp Kit (Beckman Coulter), amplicon libraries were sequenced using an Illumina MiSeq instrument (Illumina, San Diego, California, USA) according to the standard protocols of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The sequencing reads were deposited in the NCBI database (accession number: SUB12023817). The quality control of

original sequencing data was performed with fastq software (https://github.com/OpenGene/fastp, version 0.19.6). The sequencing reads were filtered and trimmed with quality > 20. The merged paired reads were spliced into a sequence with a minimum overlap length of 10 bp according to the overlapping relationship between paired-end reads. The maximum mismatch ratio allowed for an overlap area of splicing sequences of 0.2, and non-conforming sequences were screened. The allowed number of mismatches to the barcode was 0, and the maximum number of primer mismatches was 2. The amplicon sequencing reads were filtered, dereplicated, and merged using the DATA pipeline package (version 1.12) in R version 3.5.0 (R Development Core Team 2018). The amplicon sequence variants (ASV) were assigned from the sequencing data via primer-specific taxonomy classifiers (Silva132 at 99% similarity and UNITE v8 dynamic) with the QIIME2 plugin 'classify-sklearn' (Jin et al. 2022). An average of 12982 ASVs for bacteria and 78837 ASVs for fungi were produced. Bacterial and fungal sequences were analysed by the SILVA database (v.138) and UNITE (V.8.0) as reference databases, respectively (Quast et al. 2013; Callahan et al. 2016; Nilsson et al. 2019). The sequencing data were submitted to NCBI (Accession number: PRJNA946179).

Real-time quantitative PCR

The extracted DNA was used to detect the abundance of genes related to soil N reactions. qRT-PCR was performed using a 20-μL reaction system with pairs of gene-specific primers (Table S1) according to the manufacturer's instructions (Vazyme Biotech Co., Ltd, Nanjing, China). The reaction mixture contained 10 μL of 2× ChamQ SYBR Color qPCR Master Mix, 0.8 μL of forward primer (5 μM), 0.8 μL of reverse primer (5 μM), 0.4 μL 50 X ROX Reference Dye I, 2 μL of template DNA, and 6 μL of ddH_2O. The reaction conditions were as follows: initial denaturation at 95 °C for 5 min, 40 cycles of at 95 °C for 3 min, melting at 95 °C for 5 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. Vectors (pMD18-T) carrying plastids with cloned targets were measured using OD_{260} values with an ultraviolet spectrophotometer (NanoDrop2000, Thermo Fisher Scientific, USA) and converted into copies/μL. Subsequently, the plastids were serially diluted and used to plot the standard curves per functional gene (Frey and Rieder 2013).

Statistical analysis

The α-diversity indices of the bacterial and fungal communities were calculated as the Chao and Shannon indices in R (Oksanen 2011), based on the genus and phylum levels (Wickham 2009). The β-diversity indices of the bacterial and fungal communities were calculated based on Bray-Curtis dissimilarities with the 'ordinate' function of the Phyloseq package and visualised using non-metric multi-dimensional scaling (NMDS) ordinations (McMurdie and Holmes 2013). Permutational multivariate analysis of variance (PERMANOVA) was performed to analyse the difference in microbial community dissimilarity, using the 'adonis' function of the vegan package in R, based on 999 permutations in the Bray-Curtis distance matrix (Oksanen et al. 2007). Dominant bacterial and fungal phyla were defined as those with abundance > 10%, while 'other' phyla were defined as those with abundance < 1%. Linear discriminant analysis (LDA) effect size (LEfSe) was used to analyse the differences in the relative abundances of bacterial community members among different treatments using an online Galaxy application (version 1.0) (http://huttenhower.sph.harvard.edu/galaxy/). The correlation of environmental variables and microbial community composition was assessed by the Mantel test and Pearson's correlations and visualised with the 'ggcor' package in R (Huang et al. 2020). A relative abundance in taxonomic composition > 1% was used to calculate pairwise distances among samples. Environmental parameters were constructed using the Euclidean distances among the samples (Wu et al. 2021). Correlations between microbial community composition and environmental parameters were constructed with the partial Mantel test using the vegan package (9,999 permutations) (Dixon et al. 2003) and 'ggcor' packages in R. The correlation heatmap between the differential microbial family and soil enzyme activities was constructed with Pearson's correlations and visualised with the 'ggcor' package in R.

Structural equation modelling (SEM) was used to evaluate the correlations among bacterial community composition, fungal community composition, C-, N-, and P-related soil enzyme activities, soil N process-related gene expression, and soil physiochemical variables (Chen et al. 2019). Principal component analysis was performed to develop multivariate functional indices into one new parameter. PC1 was considered a new parameter to represent the combined group factors. Soil physicochemical properties included TC, TN, AK, CEC, NO_3^-, NH_4^+, pH, and OM. N-related soil enzyme activities included NR, NIR, DEA, PRO, URE, and GLS. C-related soil enzyme activities included PPO, NAG, CAT, and GU. The SEM was constructed using AMOS 21.0 (Amos Development Corporation, Chicago, IL, USA). The fit of the suitable model was evaluated using RMSEA (RMSEA ≤ 0.05) and *P*-value ($P < 0.05$). In addition, we defined the microbial members with higher relative abundance as the drought-tolerant members.

Linear mixed-effects models were constructed to evaluate the effects of sampling time, plant sex, water treatment, and their interactions on Shannon and Chao indices based on phylum and genus. Before the analysis, the homogeneity of variances was tested using Leven's test. All statistical

analyses were performed at the 95% confidence level using SPSS software (version 22.0).

Results

Root traits

Sexual variation affected plant growth in response to drought and subsequent rewetting. As shown in Fig. S1, drought reduced the root biomass, shoot biomass, and total biomass and increased the root/shoot ratio in both sexes. This decrease in biomass was greater in females than in males. Rewetting after drought stress increased the difference in the root, shoot, and total biomass between the control and drought treatment in females, but no significant effect on these parameters was found in males. Rewetting also narrowed the difference in root/shoot growth between control and drought-treated females and males to a lesser extent.

Bacterial and fungal community composition and diversity

We analysed bacterial and fungal community composition and diversity based on the ASV level (Fig. 1). Drought and rewetting did not affect the Shannon and Chao1 indexes of bacterial community in females and males (Fig. S2). Drought stress significantly decreased the Chao 1 index of fungal community in both sexes, whereas significant differences were not observed in the Chao 1 index of fungal community after rewetting. Significant shifts in bacterial and fungal community dissimilarities were detected using the Adonis test and non-metric multi-dimensional scaling (NMDS). NMDS and PERMANOVA showed that fungi formed different clusters more clearly in females than in males between the drought and control conditions as well as between the control and rewetting conditions. However, drought significantly separated the bacterial community in the rhizosphere of both sexes, whereas rewetting had less of an effect on the bacterial diversity of both sexes in the rhizosphere (Fig. 1; Table S2).

Taxonomic composition

Drought decreased the relative abundance of the Acidobacteriota (bacteria) and Basidioycota (fungi) phyla in the rhizosphere of males but not in that of females. After rewetting, the phyla Actinobacteriota and Proteobacteria were increased in the rhizosphere of males, whereas the relative abundance of Ascomycota (fungi) was reduced in the rhizosphere of females. When exposed to drought stress, the relative abundances of dominant bacterial phyla in the rhizosphere of males were generally higher than in those of females; this included the phyla Actinobacteriota, Proteobacteria, Acidobacteriota, Firmicutes, Chloroflex, and Gemmatimonadota. This difference persisted during rewetting, except for the Acidobacteriota and Firmicutes phyla. In contrast, the abundance of dominant fungal phyla was generally higher in the rhizosphere of females than in that of males; this included the phyla Ascomycota, Chytridiomycota, and Mucoromycota (Fig. 2; Table S3). Interestingly, the relative abundance of Ascomycota was lower in the rhizosphere of females than in that of males at the rewetting stage (Table S4). Interestingly, more drought-tolerant fungal members (those with increased relative abundance in the drought treatment) were enriched in the rhizospheres of both female and male plants. The fungal but not bacterial community was more easily affected by rewetting in the rhizosphere of females; the reverse was true for males, as more bacterial members showed a high relative abundance in the drought treatment (Fig. S3). Drought elevated the relative abundance of Saccharimonadaceae and Acidimicrobiia in the rhizosphere of both sexes. Rewetting did not affect the bacterial community in the rhizosphere of females but strongly affected the differential colonisation by bacterial communities in the rhizosphere of males when compared to controls (Fig. S3). For the fungal community, drought promoted the colonisation of Pseudeurotiaceae, Clavicipitaceae, Mrakiaceae, and Ustilaginaceae in the rhizosphere of females and Bolbitiaceae, Ascodesmidaceae, and Coniochaetaceae in the rhizosphere of males. Drought and rewetting changed the sexual differences in bacterial and fungal enrichment in the rhizosphere (Fig. 2). Drought induced the enrichment of A4b and PLTA13 in the rhizosphere of males, which was previously enriched in the family Gailellales, 67-14, Bacillaceae, and Azospirillales. After rewetting, the families Devosiaceae and Hymenobacteraceae were enriched in the rhizosphere of females, whereas the JG30-KF-CM4, Rokubacteriales, and WX65 classes were enriched in the rhizosphere of males. Males had a higher abundance of the fungal members in their rhizospheres, such as the families Nectriaceae, Trichocomaceae, Mrakiaceae, Mortierellaceae, and Ascodesmidaceae, relative to females. After rewetting, the families Rhizopodaceae, Piskurozymaceae, and Agaricomycetes were enriched in the rhizosphere of males, whereas the family Chytridiomycota was enriched in the rhizosphere of females (Fig. 2).

Correlation between bacterial and fungal community composition and soil properties

RDA was performed to explore the soil properties that affected bacterial and fungal communities (Fig. 3). Under drought conditions, the first two axes of the RDA explained 70.4% and 19.6% of the total variation for the relationship between soil properties and soil bacterial community, and 59.9% and 13.2% for that between soil properties and fungal

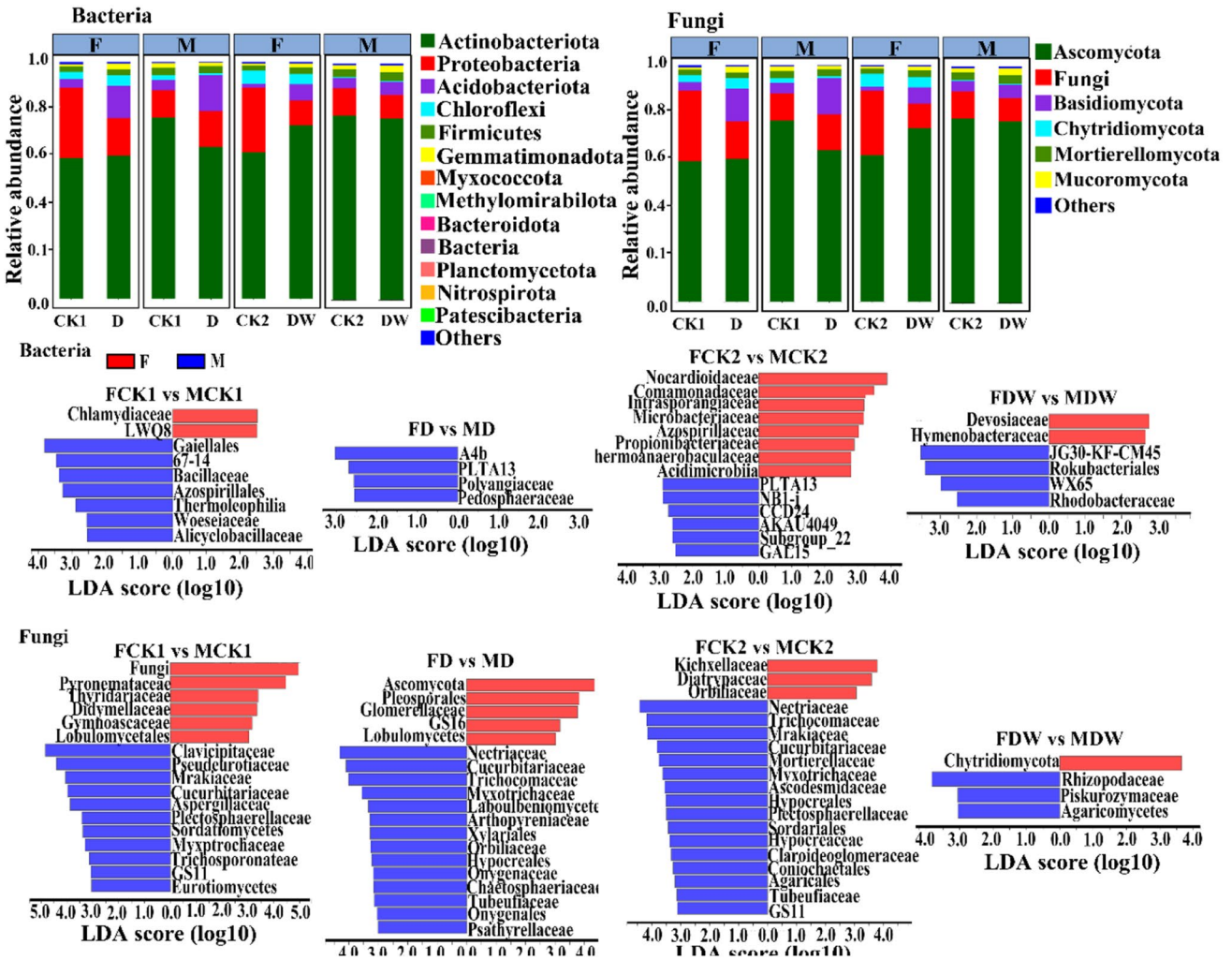

Fig. 2 Taxonomic composition and species difference analysis between sexes in the rhizospheres of *Populus cathayana* females (F) and males (M) under drought and rewetting. The dominant taxonomic components of bacterial and fungal communities are shown at the phylum level, with a minimum relative abundance of 1% species. Phyla with a relative abundance of less 1% species are indicated as 'others'. LEfSe was used to identify abundant taxa in the rhizosphere of females and males. Only taxa with LDA over 3.0 are shown. CK1, previous control; D, previous drought; CK2, control after rewetting; DW, rewetting

community. After rewetting, the first two axes of the RDA explained 52.3% and 35.9% of the total variation for the relationship between soil properties and soil bacterial community and 66.1% and 13.4% for that between soil properties and fungal community. At the end of the drought, GU, PPO, and URE could highlight the differences in bacterial community composition between females and males while PPO, NR, GLS, and NIR could highlight differences in fungal community composition between females and males (Fig. 3). After rewetting, PPO, APH, NIR, and NR regulate sex-specific bacterial community composition, and NR and NIR regulate drought-driven bacterial community composition in both sexes. In contrast, the fungal community was mainly regulated by NIR, APH, PRO, and GU in the rhizospheres of both sexes after rewetting (Fig. 3).

We used the partial Mantel test to decipher the relationships between microbial community similarities and environmental distances (Fig. 4). After the drought, the fungal community was more easily affected by soil environmental distance relative to the bacterial community in the rhizosphere of females; this difference included SWC, NNR, NIR, and *nirS*. In contrast, the bacterial community was more strongly correlated with NO_3^-, NH_4^+, MN, and *AOB* in the rhizosphere of males under drought conditions. After rewetting, the bacterial and fungal compositions showed a greater effect on regulating the changes of soil properties relative to that after the drought treatment. The fungal community showed clear correlations with NO_3^-, NH_4^+, MN, PRO, APH, *nirS*, and *nirK* in the rhizosphere of females, whereas the bacterial community was more correlated with TC after

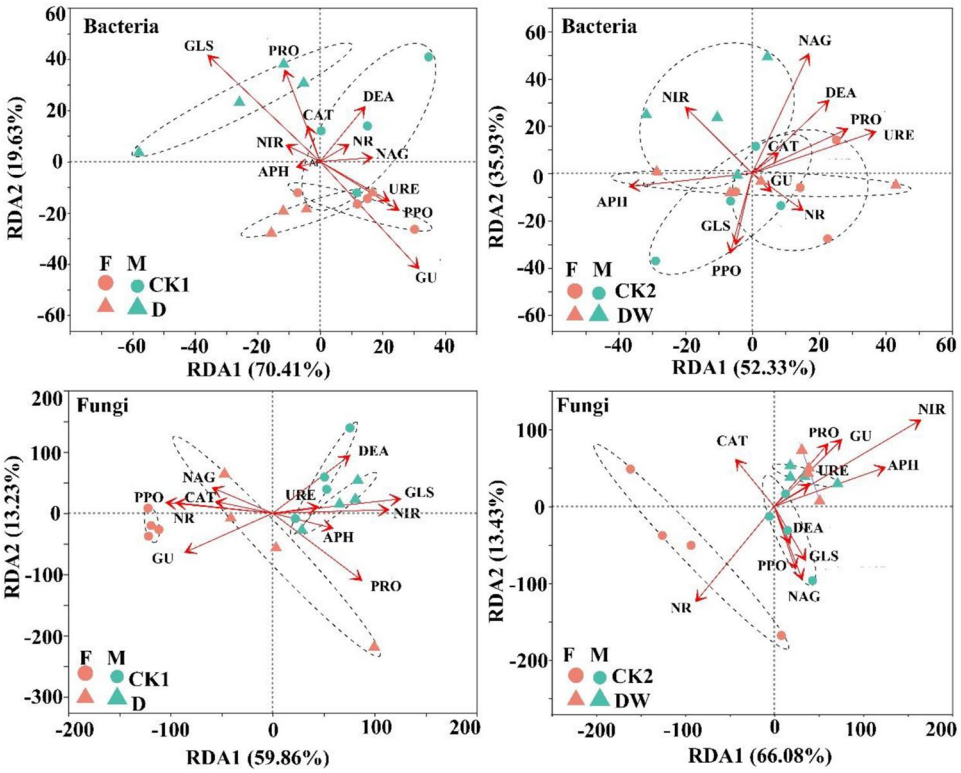

Fig. 3 Redundancy analyses (RDA) showing the correlation of microbial community components (bacterial and fungal) and enzymatic activities related to soil C, N, and P reactions in the rhizosphere of *Populus cathayana* females (F) and males (M) under drought and rewetting, analysed by redundancy analyses (RDA). Only the first two components are displayed. CK1, previous control; D, previous drought; CK2, control after rewetting; DW, rewetting. APH, acid phosphatase; URE, urease activity; NIR, nitrite reductase activity; NR, nitrate reductase activity; PRO, protease activity; PPO, polyphenol oxidase activity; GU, β-glucosidase activity; NAG, N-acetyl-β-D-glucosaminidase activity; CAT, catalase activity; GLS, glutaminase activity; DEA, denitrifying activity

rewetting (Fig. 4). In the rhizosphere of males after rewetting, correlations existed between the bacterial community and AMR and NAG and between the fungal community and TN, NH_4^+, and CEC.

At the end of the drought, microbial C and N contents were mainly driven by soil N content and its processes in the rhizosphere of females and by soil nutrient content (soil C, N, and P) in the rhizosphere of males. After rewetting, the soil physiochemical properties (except for OM and TC) and N processes had a greater effect on the microbial C and N contents in the rhizosphere of males while the relationship between soil N processes and microbial C and N was not affected in the rhizosphere of females. In addition, at the end of the drought treatment, most N reaction-related genes exhibited weaker correlations with soil nutrient and enzyme activities in the rhizosphere of females relative to that of males. The opposite trend was found after rewetting. Specifically, genes related to N fixation and denitrification were more relevant to most soil environmental distances in the rhizosphere of females relative to that of males after rewetting.

Expression of N-related genes

Drought significantly increased the expression of ammonia-oxidizing archaea (*AOA*) and ammonia-oxidizing bacteria (*AOB*) genes in both sexes, and the effect was greater in the rhizosphere of males than in that of females (Fig. 5). Drought stress downregulated the relative abundance of *nifH* and *nirK* genes in both sexes. In the rhizosphere, males had a higher relative abundance of *nifH* but a lower abundance of *nirK* compared with females. Rewetting reduced the expression of *AOA*, *AOB* (except in females), *nifH*, *nirK,* and *nirS* genes but increased the expression of *nosZ* in the rhizospheres of both sexes (Fig. 5). However, the expression of *AOA*, *AOB,* and *nirK* was higher in the rhizosphere of females than that of males, and significant differences were not observed in the expression of *nifH* or *nirS*. Finally, in the rhizosphere, the expression of *nosZ* by males was higher than that by females.

Association between the abundance of microbial community members with soil nutrient processes

We then examined the relationship between the differential abundance of microbial families and soil enzyme activities with soil nutrient reaction under drought stress and after rewetting (Fig. 6; Fig. S4). We found that the abundance of drought-tolerant bacterial members was positively associated with PRO, NIR, GLS, NAG, and APH in females and with NR, PRO, and GLS in males under drought conditions (Fig. S4). The abundance of drought-tolerant fungi was positively associated with NIR, GLS, and DEA in females and with NR, PRO, and GLS in the rhizosphere of males under drought stress. After rewetting, the rewetting-specific bacterial members (high relative abundance

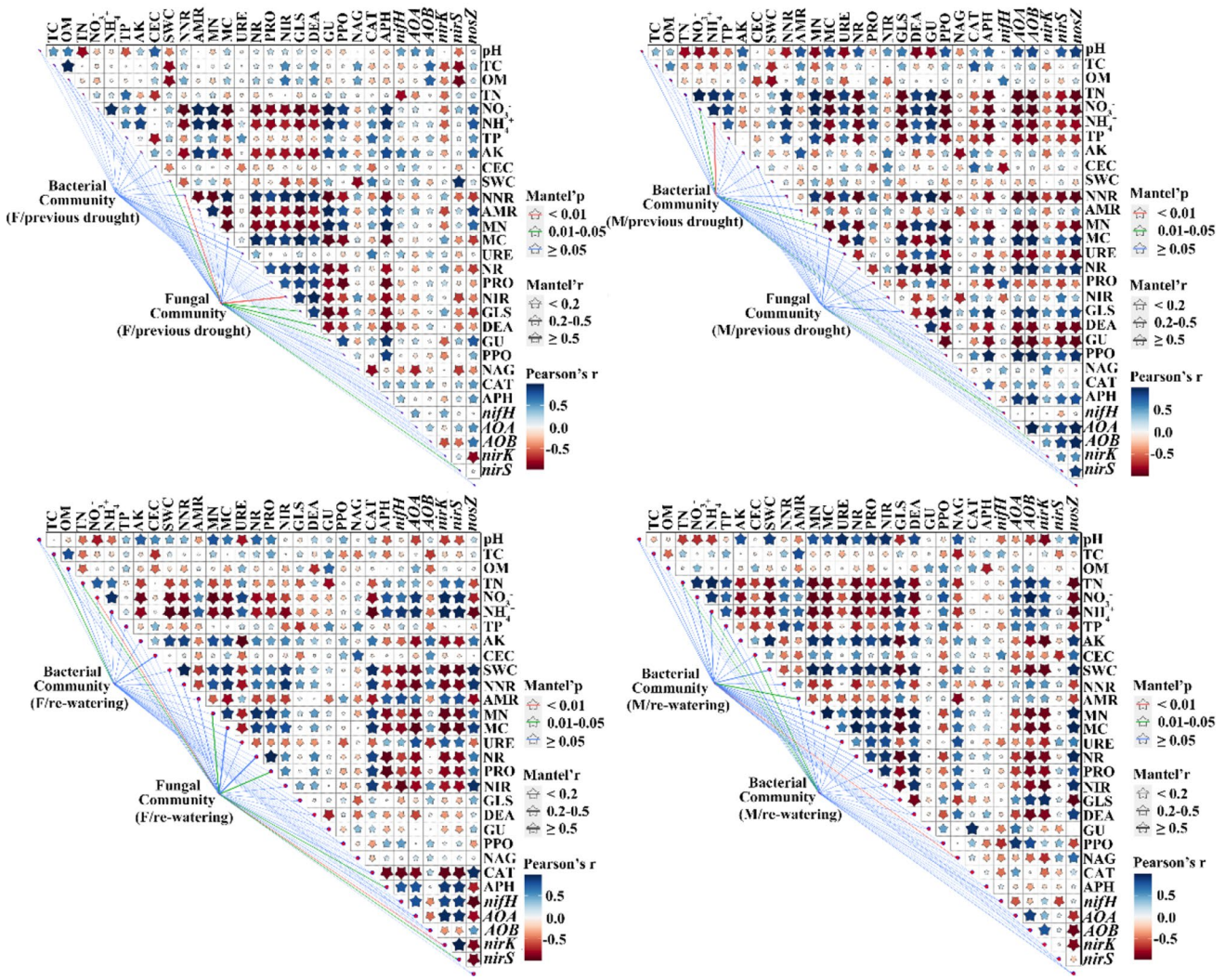

Fig. 4 Correlation of soil properties and bacterial and fungal community composition in the rhizospheres of *Populus cathayana* females (F) and males (M) under drought and subsequent rewetting. The bacterial and fungal community compositions (based on ASV level) were analysed based on the Bray-Curtis distance to each soil properties with a partial Mantel test. Soil properties were analysed based on the Euclidean distances. The line colour indicates statistical significance based on 999 permutations, and the line width corresponds to the partial Mantel's r test. The gradient of soil properties denotes Pearson's correlation coefficient. APH, acid phosphatase activity; URE, urease activity; NIR, nitrite reductase activity; NR, nitrate reductase activity; PRO, protease activity; PPO, polyphenol oxidase activity; GU, β-glucosidase activity; NAG, N-acetyl-β-D-glucosaminidase activity; CAT, catalase activity; GLS, glutaminase activity; DEA, denitrifying activity; TC, soil total carbon; OM, soil organic matter; TP, soil total phosphorus; AK, soil availability potassium; CEC; cation exchange capacity; SWC, soil water content; NNR, soil nitrification rate; AMR, soil ammonification rate; MN, microbial nitrogen content; MC, microbial carbon content

after rewetting) were positively correlated with NAG and APH in females and with PRO, NIR, DEA, and GLU in the rhizosphere of males. In contrast, the abundance of rewetting-specific fungi was positively associated with NIR and GLS in the rhizosphere of females and with PRO, NIR, DEA, and GLU in males (Fig. S3). Plant sex affected the correlation between differential microbial members and soil enzyme activities (Fig. 6). When the soil was subjected to drought, the drought-tolerant bacterial and sex-specific members were positively correlated with soil NR, PRO, GLS, and CAT in the rhizosphere of males, and with soil NIR, GLU, URE, and APH in the rhizosphere of females. Sex-specific and tolerant fungi were positively associated with soil URE, NIR, DEA, and APH in the rhizospheres of females, with soil NR, GLS, and CAT in the rhizosphere of males. After rewetting, the specific bacterial members were positively associated with NAG and PPO in the rhizosphere of females and with GLU and DEA in the rhizosphere of males. The drought legacy and sex-specific fungi were positively associated with GLS in

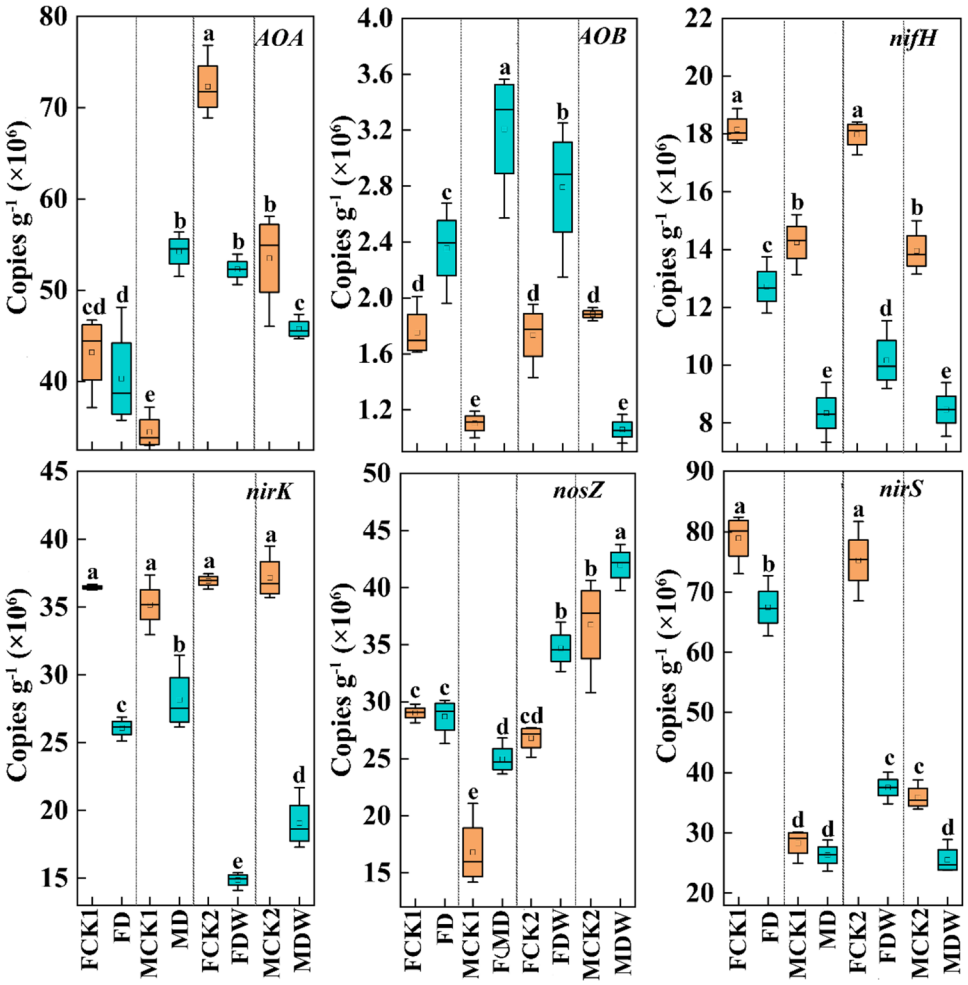

Fig. 5 Abundance of genes related to soil nitrogen cycling in the rhizospheres of *Populus cathayana* females (F) and males (M) under drought and subsequent rewetting. Bars indicate means ± SD ($n = 4$). Different lowercase letters above bars indicate significant differences at $P < 0.05$, based on ANOVA followed by Duncan's tests. Genes related to denitrification (*nirS*, *nirK*, and *nosZ*), nitrogen fixation (*nifH*), and nitrification (*AOA* and *AOB*)

the rhizosphere of females and with NR, DEA, and GLU in the rhizosphere of males.

Structural equation model

We then used SEM to identify the potential indirect and direct effects of soil physiochemical properties related to N and the microbial community on soil enzyme activities and on gene expression related to soil N dynamics (Fig. 7). After rewetting, for females, bacterial, but not fungal, community composition was directly linked to soil P mineralization and *nifH* gene abundance under drought stress, while under drought stress, bacterial community composition affected soil P mineralization via the stimulation of APH activities in the rhizosphere of females (Fig. 7). Furthermore, drought mainly affected the fungal community composition's reaction to regulated soil N via the expression of *nirK*, *nirS*, and *nosZ*, while the bacterial community composition mainly affected soil P processes via the stimulation of AP activities, as well as N processes via the expression *nifH*. In contrast, under drought stress, soil C processes were mainly regulated by soil physiological properties in the rhizosphere of males while the bacterial community composition was mainly affected by soil N processes, especially soil denitrification, which was mainly regulated by soil moisture.

Discussion

Sex-specific N preferences are driven by rhizospheric N reactions under drought

This study showed that more drought-tolerant fungi and bacteria were responsible for the greater tolerance to drought in males. Drought significantly reduces plant photosynthetic C assimilation and belowground C allocation to the soil microbiome (Hasibeder et al. 2015; Wang et al. 2021; Liu et al. 2022b), which may have a cascade effect on soil bacteria and fungi. Indeed, more tolerant bacteria and fungi were found in the rhizosphere of males than females. Moreover, fungi are more tolerant to drought than bacteria because fungi can transfer water and nutrients over long distances with hyphae (Strickland and Rousk 2010; Fuchslueger et al. 2014; de Vries et al. 2018). Drought-tolerant fungal community

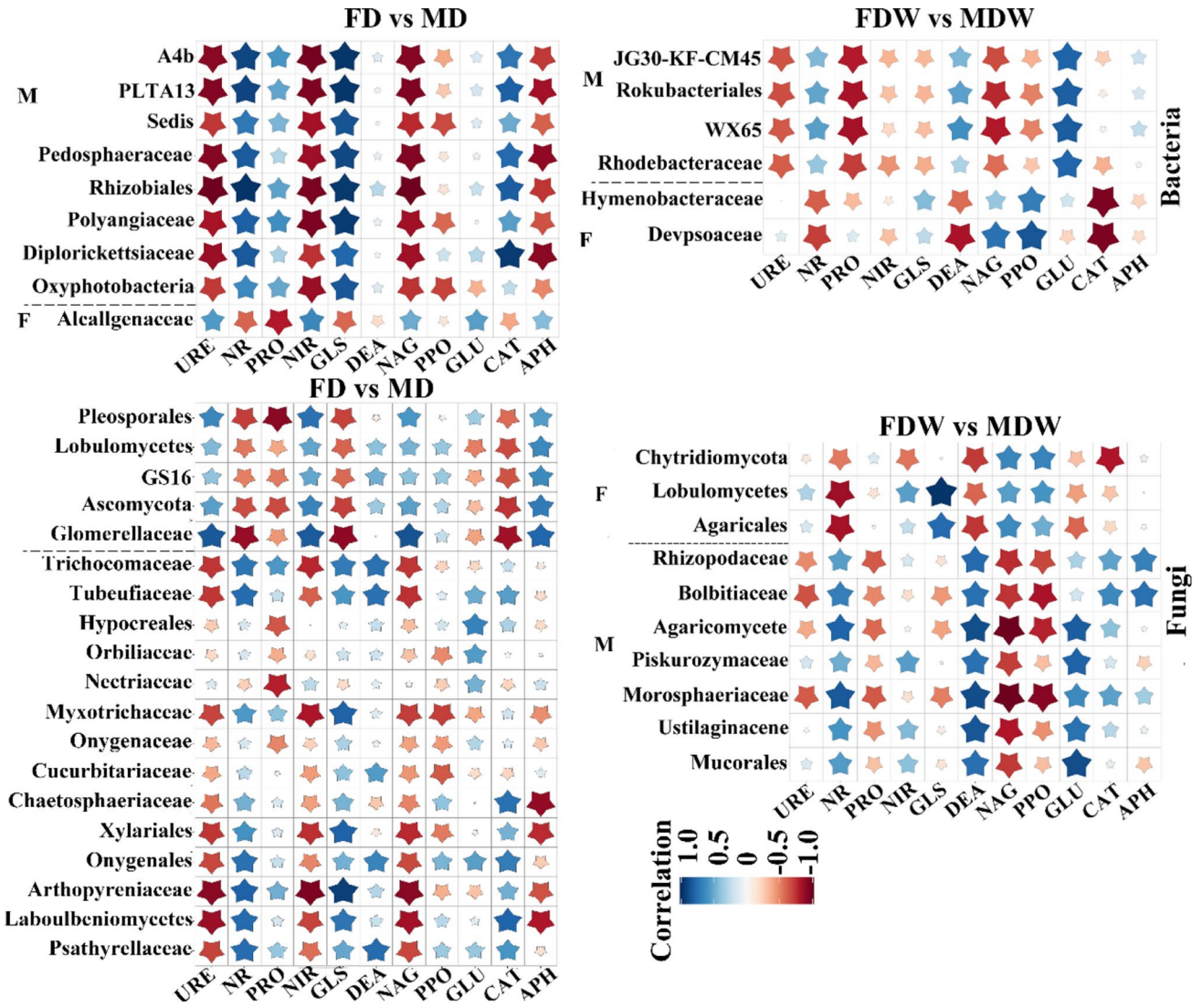

Fig. 6 Correlations between the differential fungal components and soil enzyme activities related to carbon, nitrogen, and phosphorus cycling in the rhizosphere of *Populus cathayana* females (F) and males (M) under drought and subsequent rewetting. APH, acid phosphatase activity; URE, urease activity; NIR, nitrite reductase activity; NR, nitrate reductase activity; PRO, protease activity; PPO, polyphenol oxidase activity; GU, β-glucosidase activity; NAG, N-acetyl-β-D-glucosaminidase activity; CAT, catalase activity; GLS, glutaminase activity. CK1, previous control; D, previous drought; CK2, control after rewetting; DW, rewetting

members were more abundant than drought-tolerant bacterial community members under drought conditions in the rhizosphere of both sexes, thus highlighting the critical roles of fungi in rhizosphere acclimation to drought conditions.

Plant species that prefer NO_3^--N have a greater competitive ability than those that prefer NH_4^+ (Kronzucker et al. 1997). Changes in N preference under drought stress require changes in N forms in the soil (Jia et al. 2019). The high levels of NO_3^- (but not NH_4^+) in the rhizosphere of females indicate a reduced NO_3^- availability. The increased microbial N demand and unchanged soil ammonification rate also demonstrated the reduced NO_3^- availability for females. NH_4^+ availability increased in the rhizosphere of females under drought stress as the soil ammonification rate and NR and NIR activities increased. Importantly, soil N processes were regulated by the bacterial community but not the fungal community, as shown by the significant correlation between the bacterial community and NO_3^-, NH_4^+, and *nifH* genes related to N fixation. The association between bacterial community members and the total N and NO_3^- and nitrification in the rhizosphere of males reflects bacterial community-driven NO_3^- formation. The fungal community regulated rhizospheric C processes, which may indirectly regulate soil N processes, especially considering the maintenance of the soil C:N stoichiometric ratio (Zhao et al. 2021). NAG

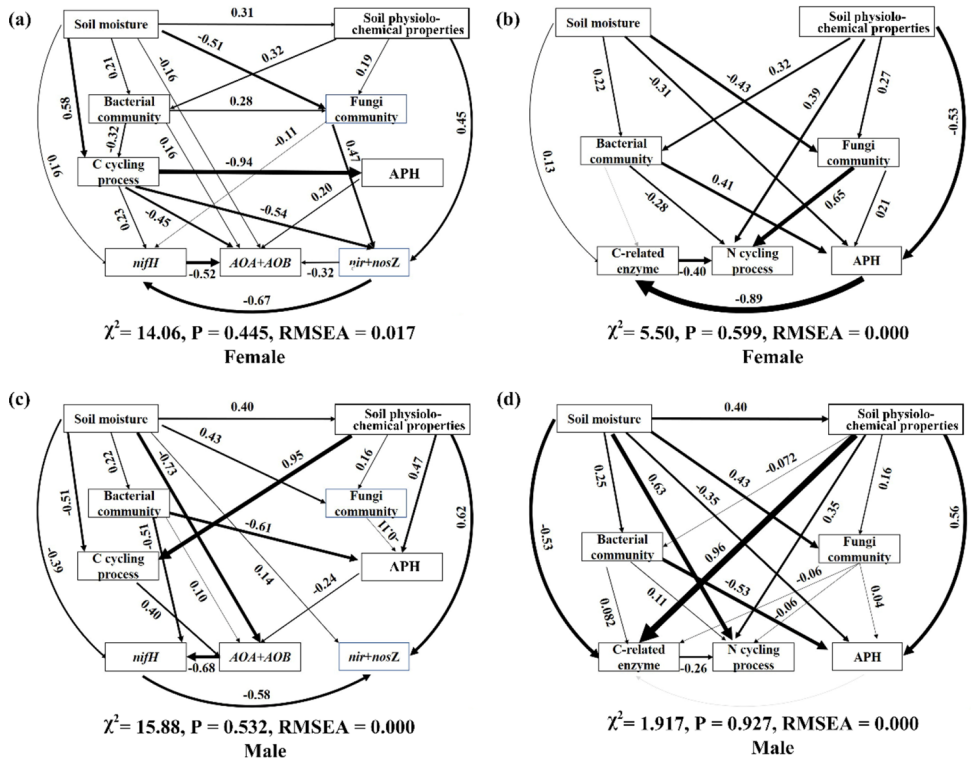

Fig. 7 Structural equation model (SEM) showing the relationship between soil enzyme activities, the abundance of genes related to nitrogen cycling, and the fungal and bacterial community composition in the rhizosphere of *Populus cathayana* females (F) and males (M) under drought and subsequent rewetting. The arrow weights are proportional to standardized coefficients which are given alongside the arrow. Multiple-layer rectangles are the first component from the soil physiochemical properties (TC, pH, TC, TN, AK, TP, NO_3^-, NH_4^+, and CEC), carbon cycling-related enzyme activities (URE, NR, NIR, PRO, CAT, PPO, and NAG) and nitrogen cycling enzymes activities (URE, NR, PRO, NIR, GLS, and DEA). APH, acid phosphatase activity; URE, urease activity; NIR, nitrite reductase activity; NR, nitrate reductase activity; PRO, protease activity; PPO, polyphenol oxidase activity; GU, β-glucosidase activity; NAG, N-acetyl-β-D-glucosaminidase activity; CAT, catalase activity; GLS, glutaminase activity; DEA, denitrifying activity; TC, soil total carbon; OM, soil organic matter; TP, soil total phosphorus; AK, soil availability potassium; CEC; cation exchange capacity; SWC, soil water content; NNR, soil nitrification rate; AMR, soil ammonification rate

enzyme activity catalyses chitin degradation and is mainly driven by fungal communities in initial chitin degradation (De Boer et al. 1999; Chung et al. 2007). The significant correlation between the fungal community and soil NAG activity suggests that fungi may favour the decomposition of chitin to product polysaccharides and polymeric compounds, which could be utilised by the bacterial community. Indeed, bacteria are unable to degrade chitin instead use secondary degradation products, such as NAG (Beier and Bertilsson 2013). Males have a higher NO_3^- demand, which is associated with low nitrification activity, as shown by low expression of *AOA* and *AOB* genes related to the nitrification process. In contrast, the preferred N form in females under drought stress was mediated by the fungal community, which was correlated with NNR and NIR activity. Many fungi (eukaryotes) show various denitrifying activities, although the occurrence of denitrification was previously thought to be limited to bacteria (prokaryotes) (Shoun et al. 2012). These results suggest that the preference for NO_3^- and NH_4^+ by females was mainly influenced by microbially mediated N reactions in the soil under drought stress.

Microbial-induced organic N mineralization limits inorganic N uptake by plants under semi-arid conditions (Huygens et al. 2016). Actinobacteria are decomposers of OM in soil and promote N reactions (Bhatti et al. 2017). Here, Actinobacteria species were more abundant in the rhizosphere of males under drought stress, implying their roles in N availability. Soil organic N mineralization also depends on extracellular proteases, such as PRO (Zhou et al. 1987; Kandeler et al. 2011). The observed drought-induced activation of PRO may suggest the roles of the bacterial community in mediating NO_3^- availability to males. In addition, plant N preferences were also dependent on soil C and P metabolism. For males, tolerance to drought was mainly achieved by bacteria-mediated N turnover (mainly nitrifying processes), soil P mineralization, and fungi-mediated litter decomposition. A sufficient

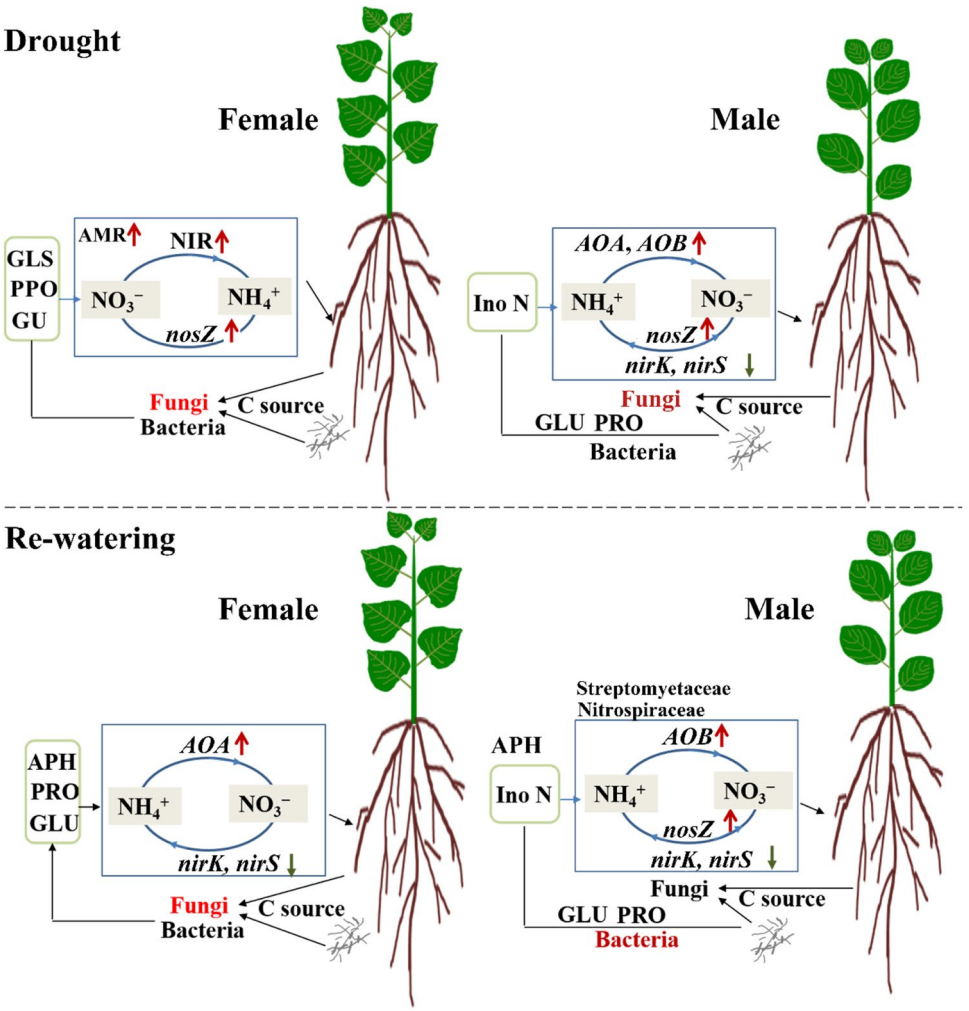

Fig. 8 Schematic model of the responses of bacterial and fungal communities to drought and rewetting and regulation of soil nutrient cycling in the rhizospheres of *Populus cathayana* females (F) and males (M). The fungal and bacterial communities were more responsive to drought stress in the rhizospheres of both sexes. Drought promotes ammonium formation in soil via microbes, which may be associated with the regulation of NNR and NIR activities, as well as the upregulation of soil nosZ genes, especially by the fungal components. Drought probably induced the bacterially mediated nitrification process, as shown by the upregulation of *AOA* and *AOB* genes but the down-regulation of *nirK* and *nirS* in the rhizosphere of males. Rewetting significantly affected the abundance of fungal components in the rhizosphere of females and of the bacterial community in the rhizosphere of males. The microbial community, especially the fungi community, was involved in the transfer of NH_4^+ into NO_3^- via the upregulation of the *AOA* gene, downregulation of the *nirK* and *nirS* genes, and changes in APH activity in the rhizosphere of females after rewetting. The bacterial community may respond quickly to rewetting after drought in the rhizosphere of males, as increased drought-tolerant bacteria, and soil N reactions (e.g., Streptomyetaceae and Nitrospiraceae)

NO_3^--N supply is a prerequisite in males for acclimation to drought stress (Fig. 8).

Rewetting mainly increases the relative abundance of the bacterial community in the rhizosphere of males

Recent studies have suggested that drought stress has a legacy effect on the composition of the soil microbial community after rewetting (Zhang et al. 2018; Canarini et al. 2021). Drought kills some cells whereas the surviving cells may be presented in a dormant state (Canarini et al. 2021). Therefore, after rewetting, the fraction of dormant-overactive microbes may increase (Meisner et al. 2018). Indeed, after the rewetting treatment, the microbial activity of the soil differed from that after the control treatment. Rewetting reshaped the bacterial and fungal communities, as shown by the differential abundance of bacterial and fungal members between the control and rewetting treatments in the rhizosphere of both sexes. Bacterial but not fungal abundance was affected by rewetting more significantly in the rhizosphere of males, suggesting that bacterial community members may

participate in the regulation of drought legacy effects. After the drought treatment, drought-tolerant bacteria, such as Streptomyetaceae species, were enriched in the rhizosphere of males. Streptomyces are gram-positive mycelial bacteria frequently reported to survive adverse conditions, such as drought stress (Esmaeil et al. 2019), and they can promote the plant growth-promoting potential by producing IAA and siderophores under normal and stress conditions (Jog et al. 2012; Palaniyandi et al. 2014). The high abundance of Streptomyces in the rhizosphere of males may promote plant survival from drought. Moreover, this study found that males preferred to recruit bacterial members related to soil nutrient reactions, such as species in the families Geodermatophilaceae, Sphingomonadaceae and Nitrospiraceae. Sphingomonas decompose persistent organic contaminants and fix atmospheric N (Adhikari et al. 2001; Feng et al. 2017). Nitrosotalea lineages account for most of the *AOA* community (Martens et al. 2009), which was also detected in our study. The relative abundance of these bacterial members in the rhizosphere of males may reflect the nutrient demand for NO_3^--N, in which the demand for inorganic N increased under rewetting, whereas the microbial N demand decreased, despite the large NO_3^- supply, allowing recovery and subsequent growth in males. In addition, fungi-mediated NAG activity also contributed to the increased soil inorganic N content in the rhizosphere of males (Fig. 8).

The rhizosphere processes mediated by fungi were more important in females after rewetting

Fungal but not bacterial abundance was more enriched by rewetting treatment in the rhizosphere of females. Likely, after rewetting, the tolerant fungal members filled the niches occupied by bacteria (Meisner et al. 2021). Poplar females have a greater nutrient demand than males (Liu et al. 2022a), and this demand is increased by rewetting, causing an increasing demand for energy and likely preference for NH_4^+ under drought shifted to the NO_3^- preference with organic N decomposition after rewetting. This may be affirmed by the correlation of the fungal community with NO_3^- levels, microbial N, PRO, and N uptake and also by the higher nitrification rate and *AOA* and *AOB* gene aabundance but *nosZ* abundance in females than in males. Soil P mineralization is regulated by phosphatases activity in response to changes in environmental factors (Nannipieri et al. 2011). The greater P demand for females was mainly regulated by the fungal community-elevated APH activity in the rhizosphere after rewetting. The bacterial community members by considering that the bacterial community was positively associated with PPO and GLS activities (Fig. 8).

Interestingly, rewetting reduced the sex-specific differences in soil nutrient reactions regulated by the microbial community and completely eliminated the fungal-regulated soil nutrient process in the rhizosphere of both sexes. More Rokubacteriales and Actinobacteria colonised the rhizospheres of males than females in drought stress. Most Rokubacteriales belong to the phylum Actinobacteria (Zhang et al. 2022), which decompose organic matter (e.g., cellulose and chitin) in soil, thereby contributing to N cycling (Khatoon et al. 2017). In addition, the more abundant bacterial members (such as JG30-KF-CM45, WX65, and Rhodebacteraceae) in the rhizosphere of males were associated with URE, PRO, and NAG, suggesting that the utilisation of OM increased after drought stress.

Conclusions

Drought did not affect bacterial and fungal diversity but did affect their abundance in the rhizosphere of both sexes. Fungal species were more tolerant to drought stress than bacteria in both poplar sexes, although drought-tolerant fungal and bacterial members were enriched in the rhizospheres of males. In the rhizosphere of females, the fungal community likely provided a C source for bacterial members by decomposing the root litter, thus enhancing their tolerance to drought stress. Both fungi and bacteria promoted the soil ammonification process, which provided more NH_4^+ relative to NO_3^- to females under drought stress. For males, tolerance to drought was mainly achieved by *AOA*- and *AOB*-mediated nitrification, P activation, and fungi-mediated litter decomposition, which provided more NO_3^- and nutrients to males to cope with drought stress. Importantly, the fungal community was more responsive to rewetting in the rhizosphere of females while the bacterial community was involved in the regulation of drought legacy effects in males. Rewetting increased fungal community-mediated N and P reactions, bacterial community-mediated C decomposition in the rhizosphere of females, and bacterial colonisation of the rhizosphere related to N availability and drought tolerance. However, the effect of drought legacy effects on soil microbial community and their roles in soil inorganic N availability must be further explored.

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Author contribution Yang Zhao was mainly responsible for data collection, analysis, and writing; Liangliang Chen, Yankai Chen and Qihang Yang contributed to data collection; and Miao Liu (the corresponding author) was responsible for the experimental design and project management.

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Declarations

Conflict of interest The authors declare no competing interests.

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