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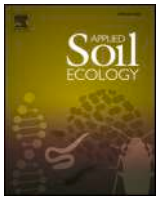

Research paper

Microbial recruitment and microbial ecological roles in soil nutrient cycling of *Populus cathayana* males and females

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ABSTRACT

Soil nitrogen (N) availability influences plant production and soil nutrient cycling. However, how it influences sex-specific microbial community composition and rhizosphere nutrient cycling in dioecious plant species is poorly understood. We examined the rhizospheric bacterial and fungal community assemble and their influences on soil nutrient cycling under different N backgrounds in 30-year-old experimental stands and a soil microbial reshaping-controlled experiment. In comparison to male trees, female trees increased fungal community diversity, and the relative abundance of taxa related to nutrient availability; elevated phosphorus (P) mobilization by increasing acidic phosphatase activity and carboxylic acid release; and decreased the counts of denitrification *nirS*, *nirK*, and *nosZ* genes at high N supply. Males increased the *nifH* gene counts related to microbial N fixation at high N supply. Low N supply increased N fixation *nifH* gene counts in the rhizosphere of females. Males decreased bacterial and fungal diversity, increased enzymatic activities related to organic N and P mineralization, and elevated soil nitrate-nitrogen levels at low N supply. Our results indicate that sex-specific responses to N availability are associated with rhizospheric bacterial and fungal community composition and diversity and their effects on rhizospheric nutrient cycling, which may explain sex-specific resource utilization and niche differentiation.

1. Introduction

Dioecious male and female plants have different reproductive costs and selective pressures, which may cause sexual specialization and niche partitioning, reinforced by changing environments (Hultine et al., 2016; Simancas et al., 2018; Liu et al., 2021a). Soil nitrogen (N) is a critical macronutrient for plant growth and community stability (Zhou et al., 2020; Niu et al., 2023). Most of the N is incorporated into organic minerals in terrestrial ecosystems, and N leaching and loss reduce N availability (Kieloaho et al., 2016; Tian et al., 2022; You et al., 2023). However, the increased application of fertilizers and the combustion of fossil fuels have increased atmospheric N deposition across most forest ecosystems (Vitousek et al., 1997; Galloway et al., 2004). These anthropic activities and complex soil processes governing N cycling may cause the spatiotemporally heterogeneous N distribution in forested ecosystems (Wang et al., 2007). Studies have found that dioecious plant males and females showed sex ratio biases across resource gradients (Hultine et al., 2016). Highly skewed sex ratios in dioecious plant species may cause population decline and species extinction, potentially affecting the structure and stability of populations with the changing climates (Petry et al., 2016). Therefore, understanding the mechanisms driving sexual spatial segregation and the coexistence of dioecious plant species under different N backgrounds is imperative.

Some soil microorganisms can link roots and soil and influence soil and plant ecology functions, such as litter decomposition, nutrient cycling, and plant species coexistence and competition (Dotaniya and Meena, 2015; Liu et al., 2023; Meng et al., 2023). Plants provide a multitude of niches for the growth and proliferation of diverse microorganisms, mainly through changes in the amount and quality of resources in the soil (Sasse et al., 2018; Zhalnina et al., 2018). Plant recruitment for rhizospheric microbiomes might be associated with soil N availability, which influences microbial ecological functions. For example, N addition increases the abundance of nitrifying and denitrifying microbes (Tian et al., 2014; Wang et al., 2018), and the reduced soil N availability promotes the colonization of some functional bacterial taxa related to root nodule and N fixation (Pueyo et al., 2021; Yazaki

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et al., 2021). Moreover, a decreased soil N supply might lead to a decrease, an increase, or have a negligible impact on the biomass and activity of saprotrophic fungi (Schroeder-Moreno et al., 2012; Jiang et al., 2014; Maaroufi et al., 2019; Du et al., 2024). Plant trait diversification in response to environmental factors has been shown to change plant features and produce new rhizospheric niches for microbial colonization (Wippel et al., 2021; Siles et al., 2023; Probst et al., 2024). Previous studies have reported that sexual dimorphism induced by soil N availability regulates leaf photosynthesis, biomass allocation, and C and N partitioning (Chen et al., 2015; Liu et al., 2021b, 2022). Such sex-specific responses to different N levels probably affect the quantity and quality of rhizospheric exudates and microbial community composition and diversity (Zhu et al., 2016; Liu et al., 2023). However, the influence of sex-specific responses of dioecious plant species to N availability on rhizospheric microbial assembly and diversity remains unclear.

A sufficient nutrient supply for plants is critical for managing soil nutrient deficiencies. Soil bacteria and fungi increase nutrient availability by releasing extracellular enzymes for organic matter mineralization and litter decomposition (Baldrian and López-Mondéjar, 2014). Rhizospheric bacteria promote plant N availability and uptake through non-symbiotic and symbiotic N fixation (Hakoyama et al., 2009; Roper and Gupta, 2016). In addition, soil fungi promote the mineralization of soil organic matter via the secretion of extracellular enzymes and increase nutrient uptake via symbiosis with host plants (Jayachandran et al., 1992). In another study, we found that rhizospheric bacteria and fungi taxa promoted N transformation and P mineralization via releasing enzymes related to P and N availability in poplars (Zhao et al., 2023). However, sex-specific N and P cycling processes regulated by soil N availability remain unclear.

In this study, we used *Populus cathayana* females and males to explore the rhizosphere bacterial and fungal community diversity and their roles in releasing available N and P with different soil N. Poplar females showed greater growth, biomass, and nutrient acquisition under resource-rich conditions but were sensitive under unfavorable conditions (Liu et al., 2024). In contrast, male plants had a higher ability to adjust physiological, morphological, and molecular traits and a better tolerance in response to stressful environments (Liu et al., 2023). However, sex-specific rhizosphere microbial assembly and its roles in regulating soil nutrient cycling under different N backgrounds are poorly understood. Therefore, we aimed to investigate (1) how plant sex affected rhizosphere bacterial and fungal community assembly under different N backgrounds; (2) how sex-specific rhizosphere fungal and bacterial communities affected soil N and P availability under different N backgrounds. Understanding microbial assembly and its roles in mediating soil nutrient cycling provides theoretical support for the sustainable management of dioecious plantations.

2. Materials and methods

2.1. Site and plantation description

The field experiment was performed at Huangyangtan farm, a state-owned forest farm in Xuanhua, Hebei Province, China (40°25′N, 115°2′E). The study area has a temperate sub-arid climate with the following conditions: 7.6 °C mean annual temperature (MAT), 365 mm mean annual precipitation (MAP), and 600–1000 m elevation. The *Populus cathayana* male and female plantations were cultivated in 1992. Poplar males and females in the field were further identified based on the flower organs in spring. Pure poplar males and females were defined when the proportion of poplar males or females was >90 %.

2.2. In situ experiments

The soil samples were collected during the growing season of the poplar plantations (Fig. 1). We collected soil samples at a depth of 0–40

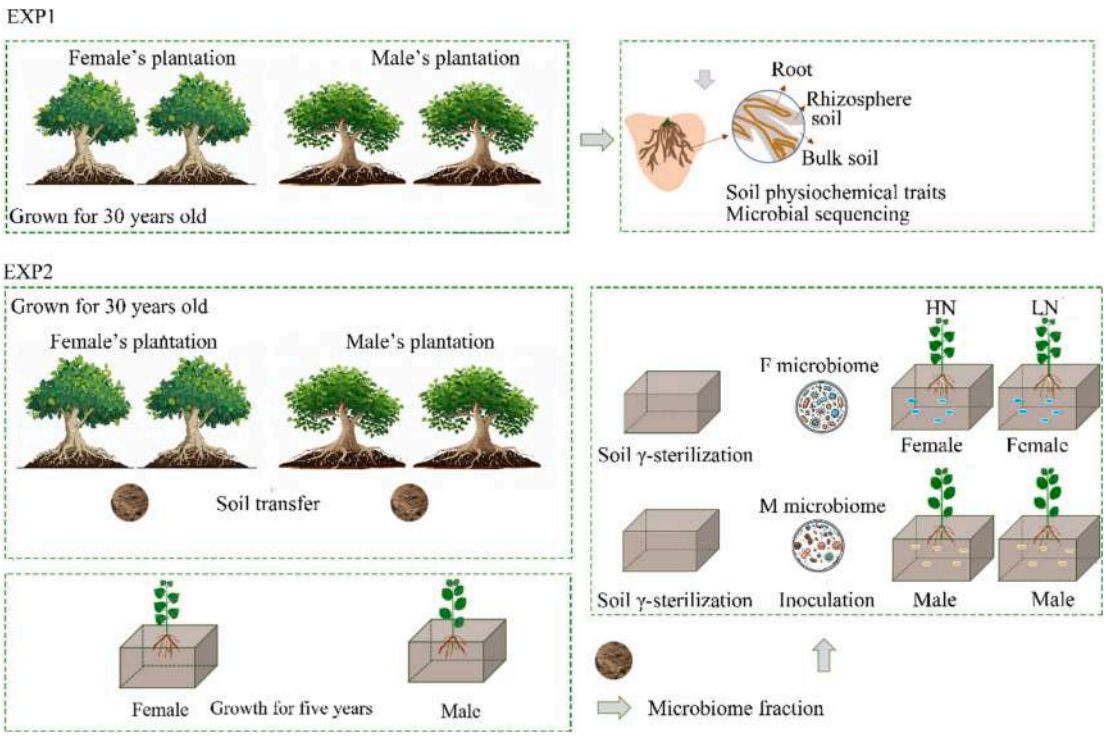

Fig. 1. Experimental design of the study. The experimental design consisted of 2 main experiments: (EXP1) in situ soil sample collection and soil conditioning from the field of 30-year-old *Populus cathayana* plantations, and (EXP2) soil microbial recruitment in the rhizosphere of *Populus cathayana* males (M) and females (F) via microbial inoculation, with high N (HN) or high N supply (LN) treatment. The inoculation soils from were prepared by mixing inoculum soils (6 % of the pot volume) from the rhizosphere of cuttings during the first stage with background soils (94 % γ-irradiated sandy soils). Soil was prepared separately according to plant sex (M cuttings were placed in soil from M plants, and F cuttings were placed in soil from F plants).

cm, 50 cm from the center of the local tree trunk. For pure males or pure female plantations, we selected four plots and four trees per plot for 32 trees (2 sex × 4 trees × 4 plots). After removing the litter layer, soil samples were collected from the four cardinal points of the trunk and pooled as a composite sample. The four soil samples from four males or females for each plot were mixed into a single composite sample and stored at 4 °C for the greenhouse experiment. One part of the combined soil samples was air-dried for soil physiochemical analysis.

2.3. Glasshouse experiments

Populus cathayana male and female cuttings were collected from 24 males and 24 females from six source stands (2 sexes × 6 source stands × 4 trees) at local farms. Therefore, a total of 24 trees were selected for the collection for cuttings. Each treatment in the glasshouse experiments contained the same number of cuttings from the source stands to ensure the independence of the experiment.

The first stage of the experiment was the soil conditioning phase (Fig. 1). The cuttings were grown in plastic pots containing 0.5 kg soil at Hangzhou Normal University in Zhejiang (30°03 N, 120°12 E). After 4 weeks of growth, 24 cuttings of each sex were selected for the experiment to ensure the health and size consistency of all seedlings used in this study. Each cutting was transferred to a plastic pot containing 10 kg soil. The pots were placed randomly within a greenhouse rather than being segregated by plant sex. Uniform cultivation methods, such as regular weeding and watering, were applied to all plants. The male and male cuttings were collected from the pure male and pure female plantations, respectively. After five years, all cuttings were harvested. The soil from each pot was pooled and mixed separately based on plant sex and stored at 4 °C in the dark. These soils were used as the inoculum soils in the second stage of the experiment.

The second experiment was conducted to obtain soil with sex-specific microbial communities by using the following process (Fig. 2): Cultivation soils were prepared by mixing inoculum soils from the first stage (6 % of the pot volume) with background soils (94 % γ-irradiated sandy soils). Soil was prepared separately according to plant sex (male cuttings were placed in soil from male plants, and female cuttings were placed in soil from female plants). One cutting was placed into each 10 L plastic pot along with 10 kg mixed soil. The background soils contained 1.75 g kg^{-1} total N, 122.56 mg kg^{-1} AP, 476.46 mg kg^{-1} AK, 106.33 mg kg^{-1} NO_3^--N, 52.69 mg kg^{-1} NH_4^+-N, and 33.32 g kg^{-1} soil organic matter. The experiment was randomized with 2 plant sexes (male and female) and 2 N availability levels (normal and deficient). Each treatment was replicated four times. Therefore, a total of 16 cuttings were selected for the pot experiment. For the N treatment, the cuttings were irrigated with a complete 8.3 mM NH_4NO_3 (HN) or 0.83 mM NH_4NO_3 (LN) nutrient solution (200 mL) every 3 d. Each treatment was replicated 6 times. The growth conditions were as follows: a nighttime temperature of 15–18 °C, a photoperiod of 12–14 h, relative humidity of 76–81 %, and a daytime temperature of 21–25 °C. The experiment was conducted at Hangzhou Normal University in Zhejiang.

2.4. Plant and soil sample harvest

After three months, all cuttings were harvested, and the roots, stems and leaves were separated. Rhizospheric soils were carefully separated

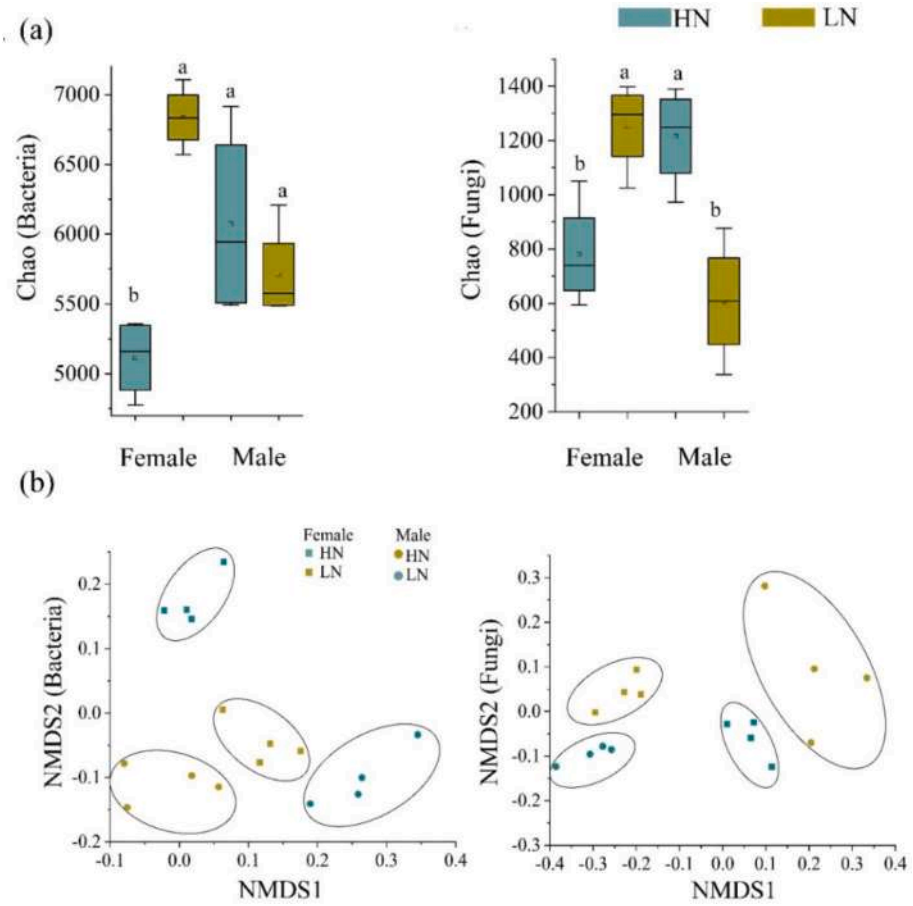

Fig. 2. Alpha diversity index (Chao) of bacterial and fungal communities (a) and nonmetric multi-dimensional scaling (NMDS) based on Bray-Curtis dissimilarity matrices of bacterial and fungal communities including all samples (b) among treatments in rhizosphere of *Populus cathayana* females and males treated with high N (HN) or low N (LN). Different lowercase letters above bars indicate significant differences among treatments at $P < 0.05$.

from the surface of the plant roots and divided into 3 parts. One part of the soil sample was air-dried naturally to measure the soil physiochemical properties, one part was used to measure rhizospheric enzymatic activities, and one part was stored at −80 °C for bacterial and fungal amplicon sequencing.

2.5. Measurement of soil chemical traits and enzymatic activities

Soil pH was measured using glass electrodes connected to a PHBJ-260 pH meter (Shanghai Leici, China) after a mixture of soil and H_2O (1:2.5) (Fu et al., 2016). For determining soil cation exchange capacity, soil samples were extracted with NH_4OAc and measured for NaOAc saturation (Laboratory, 1954). Soil total N was measured using a chemiluminescence detector (CLD-TOC) coupled with a C and N analyzer (multi C/N 3100; Jena Analytics, Jena, Germany). For soil available potassium, soil samples were digested with $HClO_4$ and H_2SO_4, followed by a measurement using plasma-atomic emission spectrometry (ICPS-7500, Shimadzu, Japan). For soil available P, soil samples were digested with H_2SO_4 and $HClO_4$ and measured using the molybdenum blue method (Olsen et al., 1954). For NO_3^--N and NH_4^+-N content, soil samples were extracted with 1 M KCl and measured using a continuous-flow analytical system (SEAL Analytical Auto Analyzer 3) (Sahrawat and Prasad, 1975; Sims and Jackson, 1971). Soil total C was measured using a chemiluminescence detector coupled with a C and N analyzer (Multi C/N 3100, Jena Analytics, Jena, Germany). For soil microbial C and N contents, fresh soil samples were fumigated with CHCl and extracted 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 calculated as the difference in C and N contents between non-fumigated and fumigated soil samples. Soil organic C was calculated as the difference in C content between total C and inorganic C (Nelson and Sommers, 1983). Fresh soil samples were incubated at 20 °C for 14 d in the dark and extracted with 2 M KCl. The net soil nitrification rate and ammonification rate were calculated as changes in NH_4^+ (Zhang et al., 2019).

For soil nitrate reductase activity (Nowak et al., 2002), soil samples were treated with KNO_3 as the substrate under anaerobic conditions and incubated at 30 °C for 24 h. The activity of nitrate reductase was expressed as reduced NO_3^--N mg g^{-1} d^{-1}. For soil nitrite reductase activity (NR) (Rao et al., 1988), soil samples were treated with $NaNO_2$ as the substrate under anaerobic conditions and incubated at 30 °C for 24 h. The activity of nitrite reductase (NIR) was expressed as reduced NO_2^--N mg g^{-1} d^{-1}. For the L-glutaminase activity (Frankenberger Jr and Tabatabai, 1991), soil samples were treated with L-glutamine as the substrate and incubated at 37 °C for 24 h. The activity of L-glutaminase (GLU) was expressed as reduced glutamine mg g^{-1} d^{-1}. For the urease (URE) activity (Kandeler and Gerber, 1988), soil samples were treated with urea as the substrate and incubated at 37 °C for 24 h. The activity of urease was expressed as reduced NH_4^+ mg g^{-1} d^{-1}. For soil *N*-acetyl-β-D-glycosaminidase (NAG) activity (Wang et al., 2015), soil samples were treated with *p*-nitrophenyl-*N*-acetyl-β-d-glucosaminidine as the substrate and incubated at 37 °C for 1 h. The activity of NAG was expressed as the released p-nitrophenol after enzyme substrate cleavage. For the assessment of denitrifying activity (RNR), soil samples were treated with KNO_3 as the substrate and incubated at 37 °C for 48 h. The denitrifying activity was expressed as the produced N_2O after enzyme substrate cleavage (Barnard et al., 2004). The N_2O content was measured using a gas chromatograph with an electron capture detector (Varian Chromatography Group, CA, USA). For soil catalase activity (CAT) (Wu et al., 2016), the soil samples were treated with hydrogen peroxide as the substrate and incubated at 25 °C for 20 min in the dark. The activity of catalase was expressed as the consumed hydrogen peroxide after enzyme substrate cleavage. For the polyphenol oxidase (PPO) activity, soil samples were treated with pyrogallic acid as the substrate and incubated at 30 °C for 2 h. The activity of polyphenol oxidase was expressed as the produced mg purpurogallin g^{-1} h^{-1} (Yang et al., 2017). For soil acid phosphomonoesterase activity (APH), soil samples were determined with the substrate of *p*-nitrophenyl phosphate and incubated at 37 °C. The soil acid phosphomonoesterase (APH) activity was expressed as the reduced *p*-nitrophenyl phosphate content.

2.6. Rhizospheric organic acid measurement

Fresh rhizospheric soil samples were extracted with deionized H_2O, filtered using 0.22 μm syringes, and freeze-dried with a vacuum freeze drier. Subsequently, the freeze-dried pellets were resuspended in 200 μL methanol internal standard extract followed by centrifugation at 12,000*g* for 10 min at 4 °C. Organic acids were determined by injecting 50 μm extracted solution into an ultra-performance lipid chromatography device (SHIMADZU extra X2) and an Applied Biosystems 4500 QTAP. Data are expressed as nanomoles of organic acids per gram of fresh soil. The analytical conditions were as follows: flow rate, 0.35 mL/min, and column temperature, 40 °C.

2.7. Real-time q-PCR analysis of N-related genes

About 500 mg of soil samples were used to extract DNA with PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted DNA was checked for quality and quantify by a nanophotometer (Implen, GmbH, München, Germany) and verified by electrophoresis in agarose gel. After that, soil DNA samples were quantified by real-time q-PCR (fluorescence quantitative PCR instrument, ABI7300, Applied Biosystems, American) with ChamQ SYBR Color qPCR Master Mix (2×) kits (Vazyme Biotech Co., Ltd., Nanjing, China), following the manufacturer's protocol with the primers given in Table S1. The q-PCR was conducted under the following conditions for 16S rRNA genes: initial denaturation 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, each for 40 cycles. The melting curve was collected when the whole reaction was kept at 65 °C to 95 °C with 0.2 °C increment at 0.05 s. The absolute quantification of gene copies was performed based on the construction of a standard curve. All detected gene fragments were cloned into the pMD18-T vector (2692 bp) followed by the transfer into *Escherichia coli* cells. The plasmids containing the fragment were verified and selected. Each plasmid concentration was set by 10-fold gradient dilution and subsequently used to construct a standard curve. The standard solution was set for 10^{-2} to 10^{-8} dilutions of *nifH* and *AOA* standards, 10^{-1} to 10^{-8} dilutions of *nirK* and *nirS* standards, 10^{-3} to 10^{-8} dilutions of *AOB* standards to prepare standard curves, upon the results of the pre-experiments. The gene copy number was calculated per ng of DNA per gram of soil.

2.8. Sequencing processing and bioinformatic analysis

The extracted DNA samples were also used to sequence processing. The bacterial community was assessed by amplification of the V3–V4 region of 16S ribosomal DNA, using the primers 338F: 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′ (Huse et al., 2008; Mori et al., 2014). The fungal community was assessed by the amplification of the internal transcribed spacer (ITS2) region, using the primers ITS3F: 5′-GCATCGATGAAGAACGCAGC-3′ and ITS4R: 5′-TCCTCCGCTTATTGATATGC-3′ (White et al., 1990). The template DNA was amplified using a 25 μL reaction system containing 0.5 μL template DNA, 5.2 μL SYBR Green and ROX mixture (2× Takara Bio Inc., Shiga, Japan), 0.25 μL of each primer (10 μmol L^{-1}), and 3.8 μL nuclease-free water. The PCR products were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified with the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After that, PCR products were pooled and sequenced using an Illumina MiSeq instrument (Illumina, San Diego, California, USA) (sequencing length of PE300). Sequencing reads were quality trimmed by the Quantitative Insights Into Microbial Ecology (QIIME2; version 2023.9) pipeline (Bolyen et al., 2019). The low-quality sequences were filtered with a

sequence length of <150 bp, average Phred scores of <20, and sequences of mononucleotide repeats >8 bp. Sequences with ambiguous base were removed. The paired-end reads were assembled using FLASH (version 1.2.11) (Magoč and Salzberg, 2011). Sequences were quality-filtered, denoised and merged using DADA2 (version 1.10.0) (Callahan et al., 2016). The amplicon sequence variants (ASVs) were obtained using the "dada2" algorithm. Raw DNA sequence files and associated metadata were deposited in the NCBI data bank with the accession number PRJNA1155203.

2.9. Statistical analysis

Soil physiochemical properties and plant biomass were analyzed using analysis of variance (ANOVA) with a significance level of $P < 0.05$. The effects of plant sex, N treatments, and their interactions on APH, URE, NIR, NR, PPO, BG, NAG, CAT, GLU and RNR activities were analyzed using two-way ANOVA. The α-diversity index (Chao index) based on bacterial and fungal ASV was calculated by Mothur v1.48.1 (Schloss et al., 2009), with Chao1 estimator (http://www.mothur.org/wiki/Chao). The results were assessed by the two-tailed Wilcoxon rank sum and boxplots were visualized using the '*ggplot2*' package (Wickham, 2009). The β-diversity of bacterial and fungal communities was analyzed using nonmetric multi-dimensional scaling (NMDS) based on Bray-Curtis dissimilarity by using the "ordinate" function of the R phyloseq package (McMurdie and Holmes, 2013). The relationships between the bacterial and fungal community compositions and environmental factors were analyzed using redundancy analysis (RDA) with the rda function using the vegan package of R software (version 2.6-8) (Oksanen et al., 2024). The differences in rhizospheric soil microbial community dissimilarity among different treatments were compared with the permutational multivariate analysis of variance (PERMANOVA) statistical tests using "adonis" in a vegan R package (Oksanen et al., 2024) with 999 permutations ($P < 0.05$). The differences in the relative abundance of bacterial and fungal taxa between low N and high N supply were analyzed using the linear discriminant analysis (LDA) effect size (LEfSe) algorithm (Galaxy web application, http://huttenhower.sph.harvard.edu/galaxy/) (Segata et al., 2011) in the rhizosphere of male or female trees (Wilcoxon rank-sum test, P-value < 0.05, LDA score > 3.5). We also used the Wilcoxon rank-sum test (P-value < 0.05) to compare the differences in the relative abundance of dominant bacterial or fungal phyla and significance among all treatments. P values were FDR (false-discovery rate)-corrected for multiple hypothesis testing.

3. Results

3.1. Bacterial and fungal community diversities

Low N supply increased the Chao1 diversity index of bacterial and fungal communities in the rhizosphere of females relative to high N supply (Fig. 2a). Low N reduced the Chao1 index of the fungal community but did not affect that for the bacterial community (Fig. 2a). Sex-specific differences in the Chao1 index of bacterial and fungal communities depended on soil N availability: the rhizosphere of males exhibited higher α-diversity values than that of females at high N supply but comparatively lower values at low N supply (Fig. 2a). Additionally, NMDS analysis revealed that the bacterial and fungal community compositions (β-diversity) were influenced by plant sex and soil N availability (Fig. 2b). Soil N availability altered the composition of the bacterial community in the rhizosphere of males but not the composition of the fungal community in females (Fig. 2b).

3.2. Composition and distribution pattern of the dominant bacterial and fungal taxa

The top three bacterial phyla in the rhizosphere of males and females belonged to Proteobacteria, Actinobacteria, and Chloroflexi (Fig. 3a, b). However, the proportions of Actinobacteria and Chlorflexi abundance relative to total bacterial read abundance were higher in the rhizosphere of females (22 and 19 %, respectively) than in that of males (18 and 12 %, respectively) ($P < 0.05$). Females (8 and 8 %, respectively) had a higher abundance of Firmicutes and Desulfobacterota reads than males (5 and 5 %, respectively) in the rhizosphere (Fig. 3) ($P < 0.05$). The fungal Ascomycota phyla had the highest proportion among all phyla in the rhizosphere of both poplar sexes, with a relative abundance of 82–86 % within each poplar sex, and the abundance of this taxa was higher in males than females (Fig. 3c, d) ($P < 0.05$). Males increased the fungal proportion of Mortierellomycota but decreased that of Chytridiomycota phyla compared to females.

Soil N availability affected rhizospheric bacterial and fungal abundance in two poplar sexes (Fig. 3). Most of the dominant bacterial phyla increased the abundance in females at low N supply compared to high N supply, and such N effects were greater in females than males ($P < 0.05$). Low N supply elevated the abundance of most fungal phyla in the rhizosphere of females and decreased the abundance of massive fungal phyla in males.

3.3. Taxonomic composition of the bacterial and fungal communities

The linear discriminant analysis effect size analysis (LEfSe) was used to compare the bacterial abundance from phyla to genera between plant sexes, and between soil N supply levels (Fig. 4). The Micrococcaceae family and *Arthrobacter* genus were predominant in the rhizosphere of females, and the *Paenibacillus* genus and Phodocyclaceae family were enriched in the rhizosphere of males (Fig. 4a). The fungal Ascomycota phyla were abundant in the rhizosphere of females, and males increased the enrichment of *Sphaerosporella* genus and Mortierellaceae family in the rhizosphere (Fig. 4b). High N promoted the enrichment of *Arthrobacter* genus and Micrococcaceae family but reduced that of the Gaiellales order in the rhizosphere of females (Fig. 4c). For males, the Xanthomonadaceae and Geimincoccaceae families, the *Pseudoxanthomonas* genus showed greater abundance at high N supply, and the Geobacteraceae family was predominant at low N supply (Fig. 4d). For fungi, low N supply elevated the abundance of *Heydenia*, *Pseudogymnoascus*, and *Tausonia* genera in the rhizosphere of females and the predominance of *Geopora* and *Peziza* genera in males (Fig. 4e, f).

Next, we explored the difference in relative abundance of the dominant bacterial and fungal phyla among treatments. Low N supply elevated the relative abundance of Methylomorabilota and Gemmatimonadota phyla in the rhizosphere of females. In contrast, the abundance of Latescibacterota phyla was decreased by low N in males (Fig. S2a). In the rhizosphere of females, the abundance of the dominant fungal taxa, Ascomycota and Mucoromycota phyla was increased by high N supply. Still, low N decreased the abundance of the Zoopagomycota and Basidiobolomycota phyla (Fig. S2b). There was no significant difference in the abundance of the dominant fungal phyla between two N treatments in the rhizosphere of males.

3.4. Rhizospheric physiochemical traits, microbial C and N, and rhizospheric organic acid content

Low N supply induced a 4.5-fold increase in rhizospheric NO_3^--N content in females but did not affect it in males (Fig. 5a). Low N increased rhizospheric NH_4^+ levels in both sexes. This effect was greater in females than males (Fig. 5b). Soil nitrification rate in the rhizosphere of females decreased by 25 % at low N supply. N applied levels did not affect this parameter in the rhizosphere of males (Fig. 5c). Low N supply elevated ammonification rates by 37 % and 35 % in the rhizosphere of females and males, respectively (Fig. 5d). Low N supply decreased soil pH and available P. Such an effect was greater in poplar males than females (Fig. S1). Males had the lowest pH and available P at low N supply. There was no difference in microbial C and N contents between

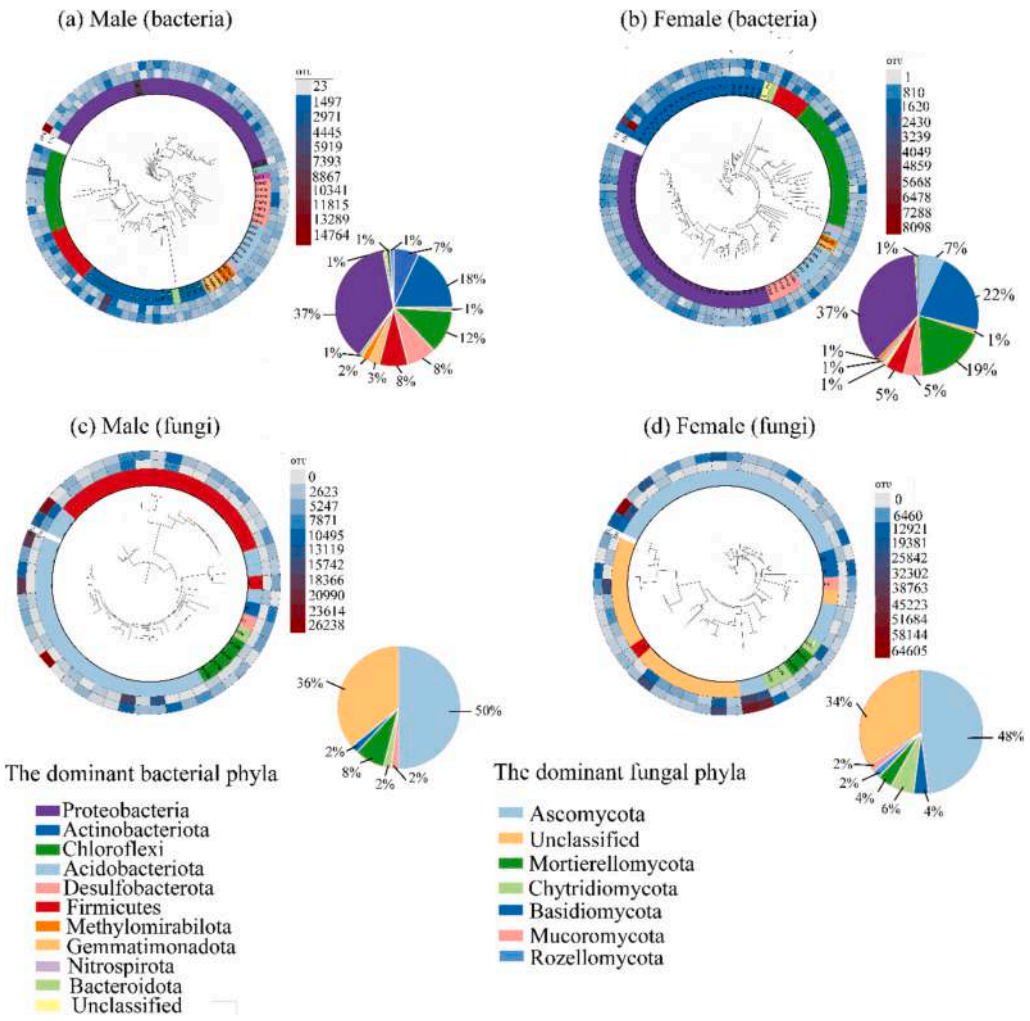

Fig. 3. Phylogenetic relationships, taxonomic composition, and relative abundance in rhizosphere of *Populus cathayana* males (a) and females (b) with high nitrogen N supply or low N supply. The phylogenetic tree was constructed using taxa with a relative abundance of >0.5 %. The colors of the outermost 2 circles represent the relative abundance of the dominant ASVs, and the inner circle represents the dominant microbial phyla.

plant sexes at high N supply (Fig. S1). Low N increased the microbial N content in both sexes and decreased microbial C content.

Plant sex and soil N supply affected organic acids, succinic acid, fumaric acid, malic acid, and lactic acid contents in the rhizosphere (Fig. 5e–h). Females had higher succinic acid, malic acid, and fumaric acid content than males, regardless of soil N availability. In addition, low N supply decreased the lactic acid content in both sexes. This effect was greater in females than males (Fig. 5h). The malic acid content increased for females and decreased for males by low N supply (Fig. 5g). Sex-specific differences in lactic and fumaric acid contents were greater at high N supply than at low N supply (Fig. 5f, h). However, the malic and succinic acid contents increased more in females than males at low N supply (Fig. 5e, g).

3.5. Soil enzymatic activities

Plant sex and soil N application did not affect rhizospheric NR, RNR, and NIR activities (Table 1). The GLS, URE, CAT, and PPO activities were higher at high N supply than at low N supply in the rhizosphere of males, but these parameters were not affected by soil N levels. Low N decreased BG and APH activities in the rhizosphere of poplar females, but increased APH activities did not affect BG in males. Soil N application did not affect rhizospheric NAG activities in females but increased this value in the rhizosphere of males. Under high N supply, poplar females had higher BG, APH, and NAG activities than males, while there was no significant difference in these three parameters under low N supply (Table 1). Females can elevate GLS and CAT activities compared to males under low N supply, but there was no difference in GLS and CAT activities between sexes under high N supply. In contrast, males increased URE and PPO activities under high N supply but lowered these two parameters under low N supply when compared to low N supply (Table 1).

3.6. Plant biomass

Low N supply decreased root and total plant biomass in poplar males and females (Fig. S3). Root and total plant biomass decreased by 52 and 48 % in poplar females and by 40 and 68 %, respectively, in males. Females had higher root and total biomasses than males, regardless of soil N availability (Fig. S3).

3.7. Correlation of soil physiological and enzymatic activities with bacterial and fungal community composition

Rhizospheric soil properties and enzymatic activities showed great variability across plant sexes and soil N availability (Fig. 6). The first two axes of the RDA grouping of all the soil properties accounted for 67 and 66 % of the variance, respectively (Fig. 6a). The rhizospheric enzymatic profiles from females showed greater potential for allocation toward APH and BG activities with bacterial and fungal community

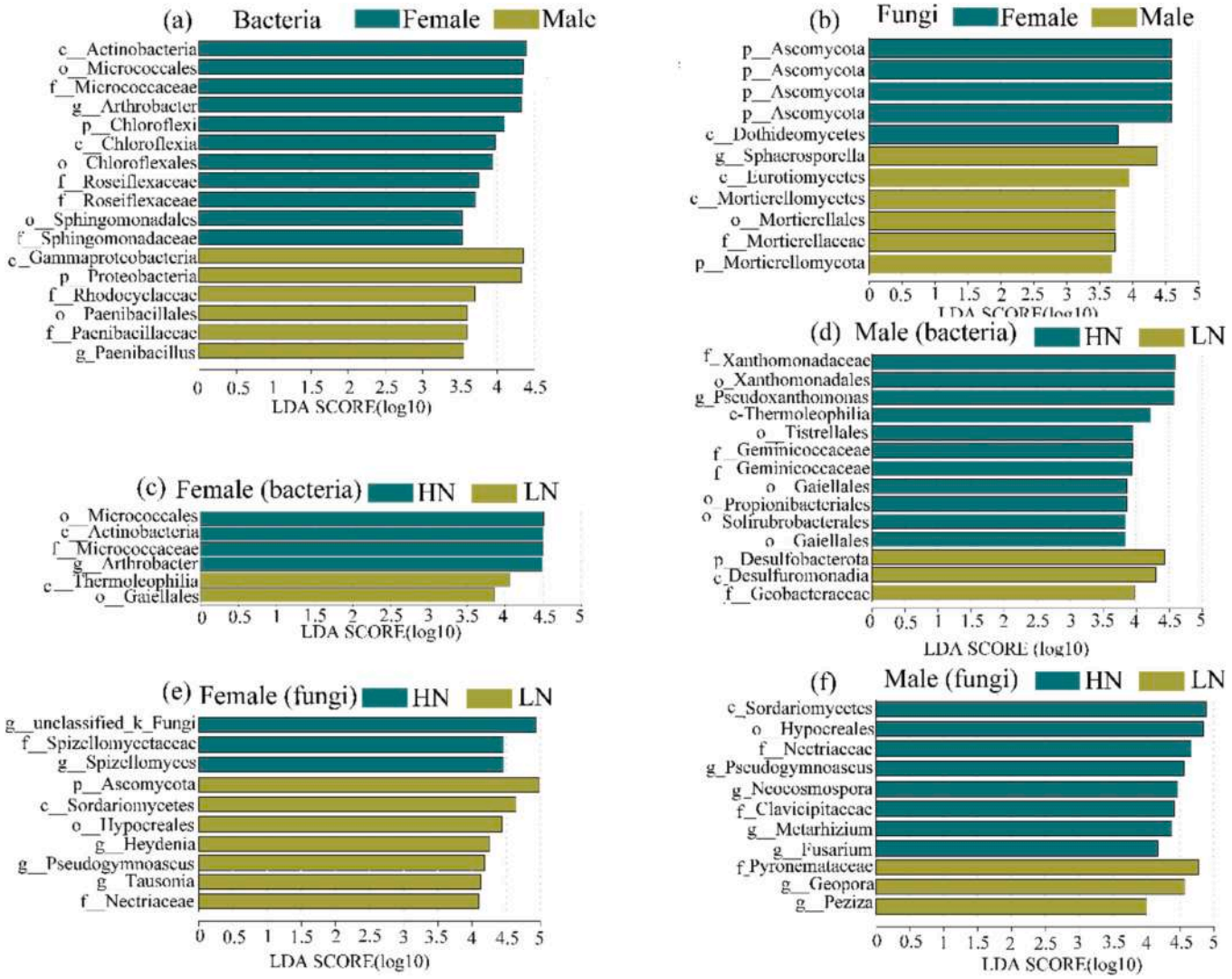

Fig. 4. Linear discriminant analysis (LDA) Effect Size analysis identified the differentially bacterial (a, c, d) or fungal taxa (b, e, f) between plant sexes (a, b) and between nitrogen (N) (c–f) treatments in rhizosphere of *Populus cathayana* males (d, f) and females (c, e) at high N supply (HN) or low N supply (LN). The color blocks indicate taxa enriched under different treatments. Only taxa with LDA > 3.8 between plant sexes and >4 between N treatments are shown.

compositions (Fig. 6a–d). NAG, URE, and NR showed a greater correlation with bacterial and fungal community composition in males than in females. Moreover, total P and CEC largely explained the rhizospheric bacterial community composition of females, and the bacterial community composition in males was mainly positively affected by soil N forms and soil pH (Fig. 6d). Fungal community composition was positively associated with CEC in the rhizosphere of females and the total C and available K of males.

3.8. Copy counts of N-related genes

Low N availability significantly decreased the copy counts of ammonia-oxidizing archaea (*AOA*) and ammonia-oxidizing bacteria (*AOB*) genes in the rhizosphere of males. It did not affect those parameters in females (Fig. 7a, b). Low N increased the copy counts of the *nifH* gene in the rhizosphere of females and decreased this parameter in males (Fig. 7c). Low N supply decreased the copy counts of *nirS* and *nirK* genes, and the effect was greater in the rhizosphere of males than in that of females (Fig. 7d–f). At low N supply, the copy counts of *nosZ* increased in the rhizosphere of females and decreased in males.

4. Discussion

4.1. Sex-specific rhizospheric bacterial and fungal community recruitment

We used the soil microbiome inoculation experiment to evaluate the bacterial and fungal assembly process at low N and high N supply. This study found that poplar males and females recruited different rhizospheric bacterial and fungal taxa at different N supply levels. Soil N availability affected plant photosynthetic C assimilation and belowground C allocation, and these effects varied by plant species and genotypes (de Ávila Silva et al., 2019), which may have a cascade effect on soil microbial recruitment (Gao et al., 2019). Studies have suggested that plant sexes respond differently to environmental stressors and have shown sex-specific microbial community diversity and composition (Lan et al., 2024). Our study also showed strong sex-specific fungal and bacterial recruitment in the rhizosphere, such as many unique taxa, varied community diversity, and abundant dominant taxa (Figs. 2–4). Our present study delivers a framework to understand the rhizospheric microbial assembly process via the sex-specifically microbial de novo inoculation and it provides guidelines for manipulating the rhizosphere microbiome to promote plant performance.

The α-diversity index of bacterial and fungal communities (Chao1) is

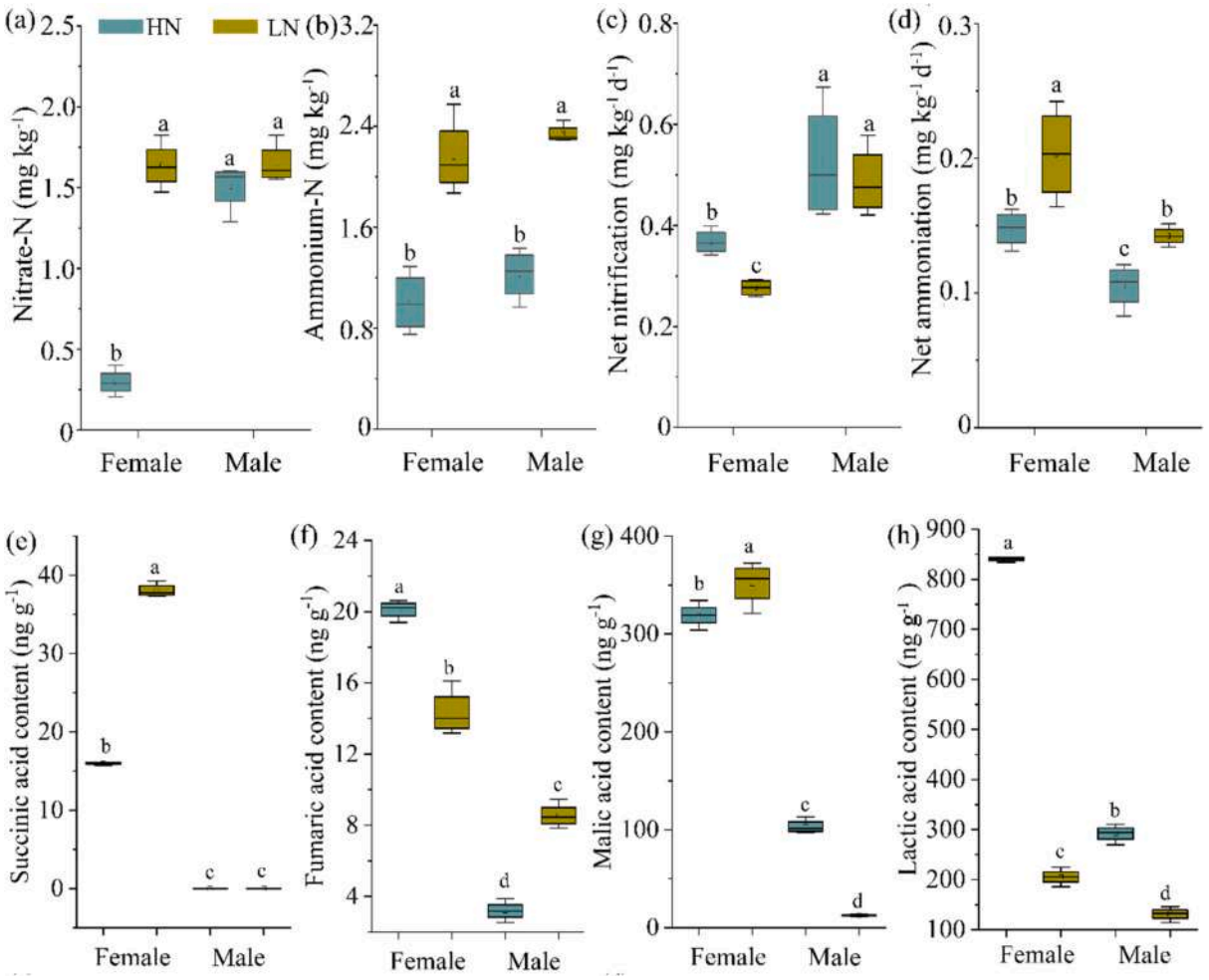

Fig. 5. Soil nitrate-nitrogen (N) (a), ammonium-N (b), nitrification rate (c) and ammonification rate (d), succinic acid (e), fumaric acid (f), malic acid (e) and lactic acid contents (h) in rhizosphere of *Populus cathayana* females and males treated with high N (HN) supply or low N supply (LN). Different lowercase letters above bars indicate significant differences among treatments at $P < 0.05$ based on ANOVA followed by Duncan's tests.

Table 1
Acid phosphatase (APH, mg kg^{-1} h^{-1}), urease activity (URE, mg g^{-1} d^{-1}), nitrite reductase activity (NIR, mg g^{-1} d^{-1}), nitrate reductase activity (NR, mg g^{-1} d^{-1}), polyphenol oxidase activity (PPO, mg g^{-1} d^{-1}), β-glucosidase activity (BG, mg g^{-1} h^{-1}), *N*-acetyl-β-D-glucosaminidase activity (NAG, U g^{-1} d^{-1}), catalase activity (CAT, U g^{-1} h^{-1}), glutaminase activity (GLU, mg kg^{-1} h^{-1}), and denitrifying activity (RNR, mg kg^{-1} d^{-1}) activities in the rhizosphere of *Populus cathayana* females and males treated with normal nitrogen (HN) and nitrogen deficiency (LN).

Sex	N treatment	NR	RNR	NIR	GLS	BG	URE	APH	CAT	PPO	NAG
Female	HN	4.48 ± 0.80a	88.5 ± 5.44a	2.42 ± 0.25a	2.75 ± 0.79ab	0.15 ± 0.03a	0.11 ± 0.002b	130.8 ± 7.31a	17.6 ± 0.16a	143.3 ± 79.67b	5.22 ± 1.16a
Female	LN	5.44 ± 0.94a	89.4 ± 5.13a	2.36 ± 0.19a	3.45 ± 0.74a	0.10 ± 0.01b	0.13 ± 0.013ab	102.5 ± 18.42b	18.9 ± 0.21a	141.1 ± 63.08b	5.34 ± 1.44a
Male	HN	4.38 ± 0.60a	89.0 ± 3.75a	2.70 ± 0.24a	2.50 ± 0.95ab	0.11 ± 0.02b	0.14 ± 0.013a	49.0 ± 14.05c	18.80.08a	190.6 ± 49.84a	7.23 ± 0.31b
Male	LN	4.41 ± 0.96a	86.5 ± 6.43a	2.44 ± 0.23a	1.98 ± 0.67c	0.09 ± 0.01b	0.05 ± 0.024c	108.9 ± 9.55b	16.7 ± 0.19b	95.3 ± 7.68c	10.42 ± 1.53a
P_{sex}		ns	ns	ns	ns	*	*	***	ns	ns	***
P_N		ns	ns	ns	ns	**	***	***	ns	**	*
P_{sex}*$_N$		ns	ns	ns	ns	ns	***	***	**	**	*

P_{sex}, sex effect; P_N, nitrogen effect; $P_{sex\times N}$, the interaction effect of sex and nitrogen. Different letters above the bars indicate significant differences between the treatments ($P < 0.05$, Duncan's test). Values are expressed as means ± SE ($n = 4$). The significant values of the two-way analysis of variance are shown as follows: ns, not significant.

* $0.01 < P \leq 0.05$.

** $0.001 < P \leq 0.01$.

*** $P \leq 0.001$.

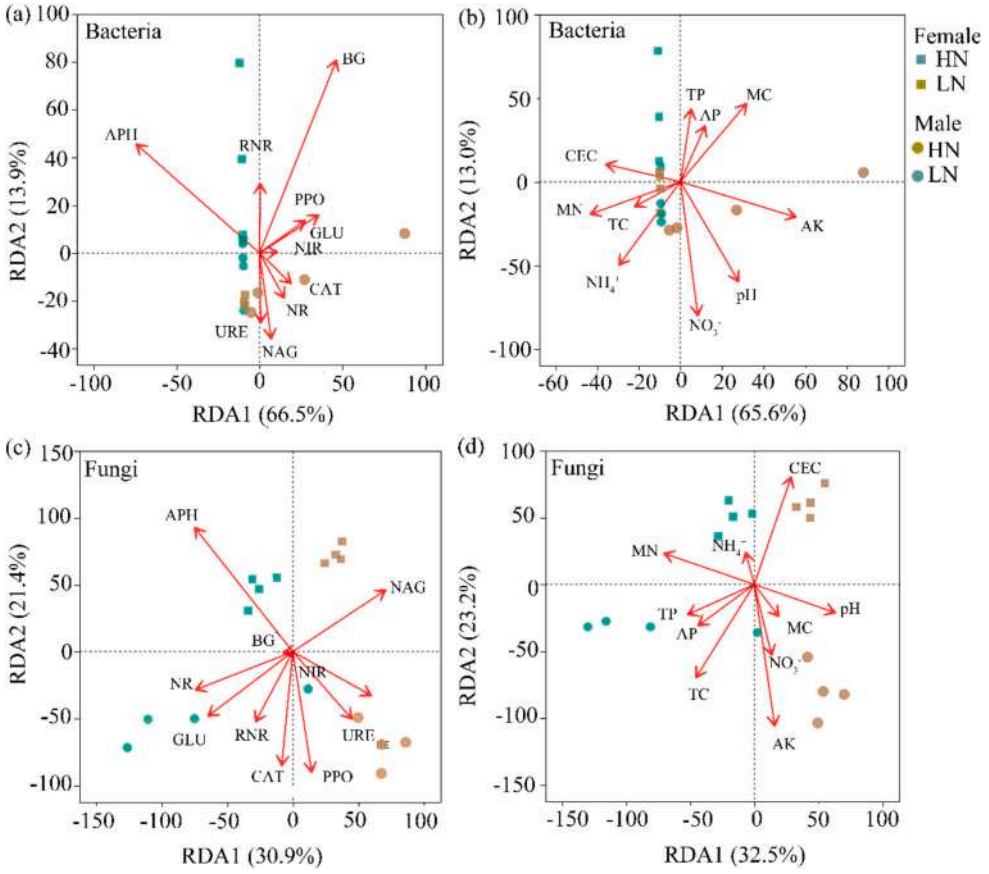

Fig. 6. Redundancy analysis (RDA) showing the relationship of microbial community composition (bacterial and fungal) with soil physiochemical factors and with enzymatic activities related to soil carbon (C), nitrogen (N), and phosphorus (P) reactions in rhizosphere of *Populus cathayana* females and males treated with normal N (HN) or N deficiency (LN). APH, acid phosphatase; URE, urease activity; NIR, nitrite reductase activity; NR, nitrate reductase activity; PPO, polyphenol oxidase activity; BG, β-glucosidase activity; NAG, *N*-acetyl-β-D-glucosaminidase activity; CAT, catalase activity; GLU, glutaminase activity; RNR, denitrifying activity; AK, available potassium; OC, organic C; MC, microbial C content; MN, microbial N content; TP, total P; AP, available P; CEC, cation exchange content.

sensitive to changes in microbial species (Shannon, 1948; Chao, 1984). Therefore, the greater increase in α-diversity at low N supply implies that a greater number of species was present in the rhizosphere of females. By contrast, the α-diversity of the bacterial and fungal communities in the male rhizosphere decreased in response to lower N supply. Reduced N supply decreases plant productivity and the associated C input, decreasing microbial diversity (LeBauer and Treseder, 2008; Liu et al., 2016). Additionally, reduced soil N availability promotes N uptake by bacteria or fungi (Johnson et al., 2015). Microbial N biomass consistently increased under N deficiency in the rhizosphere of male and female poplars, suggesting enhanced microbial N utilization in the rhizosphere of the two sexes.

Soil microbes create a linkage between roots and soil, influence the processes of litter decomposition and nutrient cycling, and provide available nutrients for their associated plant hosts (Van Der Heijden et al., 2008; Duan et al., 2023; Sawada et al., 2023). Poplar females usually had a greater demand for soil nutrients than poplar males did, which may require elevated soil nutrient availability by rhizospheric microbiome (Liu et al., 2021a). Consistently, we found that female trees preferred to recruit some taxa related to nutrient availability, such as some Actinobacteria class taxa, *Arthrobacter* genus. *Arthrobacter* is characteristic of nutritional versatility and can decompose compounds containing C and N (Jones and Keddie, 2006; Fernández-González et al., 2017). Actinobacteria enhanced plant nutrient acquisition and growth (Mitra et al., 2022a,b). Thus, these keystone taxa colonized in the rhizosphere of poplar females may be associated with fast plant growth and production. The *Paenibacillus* genus and Gammaproteobacteria class were found to be the predominant taxa in organic C and N decomposition and plant growth promotion and P solubilization (McSpadden Gardener, 2004; Das et al., 2010). Their colonization in the rhizosphere of males probably increased plant nutrient supply. The *Sphaerosporella* genus, Eurotiomycetes class, and Dothideomycetes class play critical roles in organic matter decomposition (Greiner et al., 2014; Hughes et al., 2020; Miao et al., 2022). The sex-specific recruitment for these fungal taxa emphasizes the sex-specific organic matter decomposition controlled by different functional fungi. Additionally, soil N availability affected the sex-specific recruitment of rhizosphere bacterial and fungal taxa (Fig. 1), reflecting the sex-specific response to the changed soil nutrient availability.

4.2. Females increased rhizospheric N and P availability at high N supply

Females have greater nutrient uptake and competitive ability to fulfill higher reproductive costs than males under favorable conditions (Hultine et al., 2016). Our study suggests that the larger biomass of females than males is associated with the elevated NO_3^- utilization potential and elevated soil P availability. The preference of plant species for either NO_3^- or NH_4^+ affects plant growth and competitive ability, and species that prefer NO_3^- exhibit better growth and competitive ability than species that prefer NH_4^+ (Kronzucker et al., 1997). In our study, females had higher rhizospheric nitrification activity and lower NO_3^- contents than males (Fig. 5; Table 1). The combination of soil NO_3^--leaching and plant uptake caused rhizospheric nutrient depletion and formed a nutrient concentration gradient around the roots (Akkal-Corfini et al., 2010). The increased nitrification activity in the rhizosphere of females promoted great NO_3^- accumulation. Ammonia-oxidizing

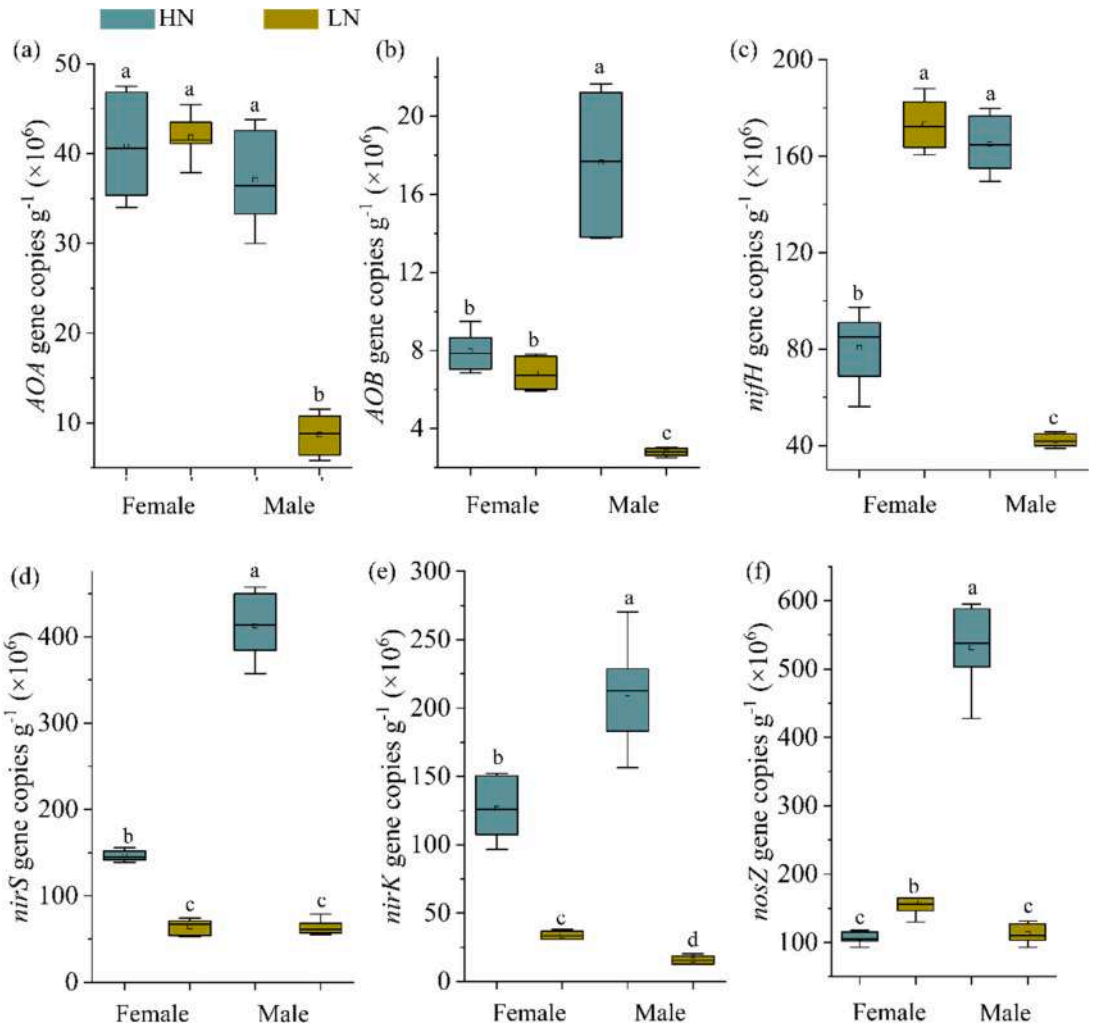

Fig. 7. Copy counts of genes related to soil nitrogen (N) cycling in rhizosphere of *Populus cathayana* females (F) and males treated with normal N (HN) or low N (LN). Data indicate means ± SE ($n = 4$). Different lowercase letters above bars indicate significant differences at $P < 0.05$, analyzed with Duncan tests after ANOVA. Genes related to denitrification (*nirS*, *nirK*, and *nosZ*), nitrogen fixation (*nifH*), and nitrification (*AOA* and *AOB*).

microorganisms can catalyze ammonia oxidation to hydroxylamine and drive soil nitrification (van Kessel et al., 2021; Sun et al., 2024). The denitrifying functional genes *nirS*, *nirK*, and *nosZ* carried by microorganisms play a crucial role in converting nitrate-N to N_2 (Zumft, 1997; Philippot et al., 2007). Although we did not observe the higher copy counts of *AOA* and *AOB* genes related to the nitrification process in females than in males, females decreased the copy counts of *nirS*, *nirK*, and *nosZ* genes related to denitrification at high N supply, which would reduce NO_3^- denitrification loss. Moreover, the pot experiment application decreased the possibility of soil NO_3^- leaching. Therefore, females' lower rhizospheric NO_3^- content than males implied great NO_3^- root uptake by females. We also found that females had a higher $^{15}NO_3^-$-N accumulation than males at high N supply, implying the preference for NO_3^- uptake by females (Fig. S3). By contrast, males increased the copy counts of the *nifH* gene related to microbial N fixation at a high N supply, implying the sex-specific N availability strategies at a high N supply in poplars.

Increased exogenous N inputs disturb the balance between N and P in terrestrial ecosystems (Elser et al., 2010). In this study, females increased soil P availability at high N supply more than males did. Rhizospheric acidification and carboxylate release are efficient P-mining strategies for mobilizing sparse P through ligand exchange and cation chelation (Mitra et al., 2022a,b; Zhang et al., 2023). In this study, at high N supply, females had a lower rhizospheric pH and higher carboxylate content in the rhizosphere than males. The ratio of malic and lactic acids relative to other organic acids detected in this study was greatest in the rhizosphere of females, suggesting malic and lactic acids were the main organic acids in the rhizosphere of females. Therefore, the decreased soil pH and increased contents of malic and lactic acids may elevate soil P availability in females at high N supply. The elevated levels of acidic phosphatase also promote organic P mineralization, an efficient strategy for releasing available P (Tarafdar and Jungk, 1987; Fox and Comerford, 1992). In our study, the higher APH activity in the rhizosphere of females than that of males may have promoted P release from organic P pools (Table 1), which may promote P availability and plant growth at high N supply. Soil microorganisms can drive P cycling via the release of enzymes (Luo et al., 2017). Consistently, soil bacterial and fungal community composition positively correlated with APH activity at high N supply (Fig. 6), suggesting the potential roles of rhizospheric bacterial and fungal communities in regulating soil P availability of females at high N supply. These results suggest that soil NO_3^--N accumulation and available P release largely explain the greater growth of females than males at high N supply.

4.3. Male enhanced rhizospheric NO_3^- availability and organic N and P mineralization at low N supply

Organic N and P pools are considered critical supplements for

fulfilling plants' N and P demands (McLaren et al., 2015; Lan et al., 2024). The enhanced organic N and P mineralization can supply available N and P, facilitating plant tolerance to N and P deficiency (Romanyà et al., 2017; Zhang et al., 2019). In this study, the rhizospheric NAG and APH activities increased in males at low N supply. NAG enzyme activity catalyzes chitin degradation regulated by fungi, and the secondary degradation products can be used by bacteria (De Boer et al., 1999; Chung and Kim, 2007). The diversity of the bacterial community was positively associated with rhizospheric NAG activity and NH_4^+ content (Fig. 6), which may be expected to supply available N under low N supply conditions. Consistently, low N increased the rhizospheric NH_4^+ content of males but did not affect the nitrification rate in males (compared to high N supply) (Fig. 5; Table 1). Poplar males typically exhibit greater nutrient utilization efficiency than females to maximize stress tolerance in low-resource habitats (Liu et al., 2021b, 2022). The higher rhizospheric organic N mineralization in males than in females increased available N production, which may enhance plant tolerance to stress caused by low N (Zhao et al., 2023). Mineralization of organic N, accompanied by organic P mineralization, has also been detected in males under drought stress conditions (Zhao et al., 2023). At low N supply, rhizospheric APH activities were stimulated more in males than females (Table 1). The increased organic N and P mineralization facilitated available N and P release. They alleviated the growth inhibition caused by low N supply in males, which may explain the low sensitivity of their root and total biomass to low N supply (Fig. S1).

Poplar females and males have been observed to change their preferences for NO_3^- and NH_4^+ in response to soil N availability (Chen et al., 2014; Zhao et al., 2023). Males increased their preference for NO_3^-, as evidenced by the enhanced nitrification activity and unchanged NO_3^- content at low N supply (Fig. 5; Table 1). Although males had lower copy counts of *AOA* and *AOB* genes than females, genes *nirS*, *nirK*, and *nosZ* in males decreased to a larger degree under low N supply than those of females. Thus, males can increase organic N mineralization and nitrification activity to supply NO_3^-, which may elevate their tolerance to N deficiency. By contrast, low N supply did not affect the abundance of *AOA* and *AOB* but did increase the copy numbers of *nosZ* in females. The denitrification-related gene *nosZ* is involved in N_2O reduction (Yoon et al., 2016). The increased copy numbers of *nosZ* in the rhizosphere of females may increase soil N loss risk. Unlike males obtaining available N from organic N, females upregulated the copy counts of *nifH* at low N supply. The *nifH* gene encoded by nitrogen-fixing bacteria is a genetic marker of N-fixing bacteria (Thaweenut et al., 2011; Lema et al., 2016). Thus, the higher *nifH* copy number in the rhizosphere of females than in males enhanced microbial N fixation potential, which may increase N availability at low N supply.

5. Conclusions

The results of this study demonstrate that poplar females and males recruited different bacterial and fungal taxa and showed different nutrient cycling processes in response to soil N supply. Females increased bacterial and fungal community diversity at low N supply but decreased the diversity of the fungal communities. Additionally, females increased NO_3^- accumulation via enhanced nitrification ability and decreased the copy numbers of *nirS*, *nirK*, and *nosZ*, which are related to denitrification at high N supply. High N supply also promoted organic P mineralization by enhancing APH activity and inorganic P mobilization through the release of organic acids and acidification in the rhizosphere of females. Thus, females had higher plant biomass at high N supply than males. In contrast, low N supply increased NO_3^- availability via the enhanced rhizosphere nitrification ability and organic N mineralization in the rhizosphere of poplar males. Moreover, males increased P availability via enhanced APH activity to mineralize organic P under low N supply relative to high N supply. Thus, poplar males enhanced organic N and P mineralization and NO_3^- availability to elevate their tolerance to soil N limitation and alleviate plant biomass reduction. This study provides a valuable reference for improving the predictions of the impact of environmental factors on the population structure and niche differentiation of dioecious *Populus cathayana*.

CRediT authorship contribution statement

Junhua Wang: Writing – original draft, Methodology, Data curation. **Liangliang Chen:** Writing – original draft, Methodology, Data curation. **Liyun Ye:** Methodology. **Yingtao Sun:** Data curation. **Miao Liu:** Writing – review & editing, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2024.105797.

Data availability

Data will be made available on request.

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