Plant Soil
https://doi.org/10.1007/s11104-023-06347-2

RESEARCH ARTICLE

# A sexual role in regulation of the assembly of bacterial and arbuscular mycorrhizal fungal communities

**Yuanjing Zhu · Tingting Dong · Fangyuan Sun · Yuxin Xiao · Qingxue Guo**

Received: 23 June 2023 / Accepted: 9 October 2023


**Abstract**

*Background and aims* Dioecious species appear to be sexually dimorphic and they present as result distinct responses to environmental settings. However, how and to what extent dioecious plants shape microbiota to their own benefit, remains unclear.

*Methods* Male and female *Populus cathayana* plants were cultivated in soils collected from different regions. The effect of a sexual role on the assembly of the bacterial community along the plant-soil continuum and the arbuscular mycorrhizal fungal community at the root endosphere and rhizosphere were studied first. The process by which phytohormones and defensive compounds of the dioecious plants influenced the endophyte community in the plant endosphere across different soil conditions was explored.

*Results* The male and female plants imposed strong pressure on the selection of keystone species and influenced the microbe-microbe interactions of bacterial communities in the rhizosphere, roots, phloem and leaves. Females harbored a more diverse and complicated bacterial community in their phloem than the males. Similarly, the composition of AMF communities in the rhizospheres and roots also differed between males and females, respectively. The male roots had significantly higher concentrations of jasmonic acid, salicylic acid, and indoleacetic acid than the female roots. The hormones were more significantly related to the relative abundance of AMF species in the female roots than in the male roots.

*Conclusion* The plant sex imposed strong regulation in plant microbiota. The different microbiota might provide external functions for dioecious plants. Therefore, more attention is required to explore the connections between plant microbiota and dioecious species.

**Keywords** Sexual dimorphism · Phytohormones · Plant microbiota · Endophytes

Responsible Editor: Stavros D. Veresoglou.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11104-023-06347-2.

Y. Zhu · F. Sun · Y. Xiao · Q. Guo (✉)
College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 311121, China
e-mail: guoqxeco@hznu.edu.cn

T. Dong
Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

## Introduction

There is convincing evidence that the assembly of microbiota is determined by host traits and environmental settings (Trivedi et al. 2021; Guo et al. 2023). Both bacteria and fungi are regulated by the host, and

Published online: 23 October 2023

which, in turn, affect the response of plant fitness to changing climates (Wagner et al. 2016). In humans, males and females are sexually dimorphic in their morphological features, levels of sex hormones, and immunity, which then regulate sex-specific gut microbiota (Kim et al. 2020; Koliada et al. 2021). Similarly, dioecious angiosperms that comprise approximately 5–6% of angiosperm species bear unisexual male or female flowers on separate plants (Renner 2014). Diverse microorganisms provide distinct life-supporting functions to extend plant fitness as they respond to different environments (Cordovez et al. 2019; Zhu et al. 2023). However, few studies have characterized differences in microbiota among the dioecious plant individuals.

Plants provide multiple microhabitats for microbes to inhabit the rhizosphere soil, surface (epiphytes), or colonize the plant issues (endophytes) (Trivedi et al. 2021; Lin et al. 2023). The plant hosts primarily impose their selective pressure on the microbiota assembly in the rhizosphere by releasing diverse types of photosynthetic carbon that are lacking in the roots and altering soil properties, such as moisture, texture, pH, and nutrient availability (Xiong et al. 2021). Zhou et al. (2022) found substantially differential metabolites in the rhizosphere soil of males and females, which were the primary factors in forming the structure of a sex-specific microbial community. Since microbes have to overcome physicochemical barriers before successfully inhabiting plant issues, the plant hosts are more selective at filtering and structuring the endophytes than they are for microbes that live in the rhizosphere (Trivedi et al. 2021). Unlike the changes in soil conditions that are influenced by external environments, such as temperature and precipitation, plant hosts provide relatively stable interior conditions to support the ability of endophytes to biosynthesize various essential compounds (Trivedi et al. 2021). Therefore, the different interior environments are determined by plant genetics, such as those of metabolites, hormones, and physiological traits, that are the primary factors that regulate the endophytic community. Males and females differ genetically, physically, and morphologically different (Charlesworth 2002, Juvany and Munne-Bosch 2015; Wu et al. 2021). It has been hypothesized that some endophytes can only inhabit male or female plant tissues because of their different abilities to overcome the physicochemical barriers and adapt to the distinct interior environments (Guo et al. 2023). However, there is still little known about how dioecious species affect their associated assembly of endophytes.

As important constituents of plant microbiota, arbuscular mycorrhizal fungi (AMF) form mutualistic interactions with plants by facilitating the uptake of water and nutrients or promoting resistance to pathogens of their hosts (Genre et al. 2020). There is a fitness cost to the plant because it needs to release signals for AMF spores to geminate and carbohydrates, so that the fungi can grow (Keymer et al. 2017). Therefore, plant species or genotypes that have differences in the activities of their roots, including the production of root exudates, have different physiological traits in their roots to interact with the AMF (Genre et al. 2020; Pei et al. 2020). Male and female poplar plants differ in their root architecture, physiological traits, and metabolites (Juvany and Munne-Bosch 2015), which may differentially impact mycorrhizal symbiosis in female and male plants (Guo et al. 2023). Vega-Frutis et al. (2015) suggested that nutrient uptake by female plants relies more on mycorrhizae and benefits more from the mutualism than that of male plants (Eppley et al. 2009; Vega-Frutis and Guevara 2009) because females invest more in the mycorrhizal symbionts than male plants invest as shown by studies of the level of AMF colonization (Vega-Frutis and Guevara 2009). However, to date, the manner by which the dioecious species affect the AMF community in the rhizosphere and within roots still remains unclear.

A recent study clearly demonstrated that the dioecious species *Populus cathayana* Rehder has a remarkable sex selection in the bacterial and fungal communities that inhabit its roots and senescent and young leaves (Guo et al. 2022). Studies have also revealed sex-specific responses to varied soil conditions that include the allocation of carbon and the production of plant hormones and metabolites (Song et al. 2019, 2020; Wu et al. 2021; Zhou et al. 2022), which could be an important driving force in the selection of microbes. In this study, we collected soils from different regions and transplanted the dioecious species *P. cathayana* to explore the role of sex in affecting the bacterial assembly along the soil-plant continuum. It was hypothesized that male and female plants could shape and regulate different bacterial communities in various plant compartments, including the rhizosphere, root, phloem and leaf. One major question of

plant microbiota research is how environmental variation influences the interactions between plant immunity and the microbiota. Since the soil conditions largely differentially affect phytohormones and defensive compounds between male and female plants (Wu et al. 2021; Zhou et al. 2022), a goal of this study was to determine how changes in these chemicals differentially influence the endophyte community in leaves and roots. An additional goal was to explore the role of sex in affecting the AMF community in the root endosphere and rhizosphere. It was hypothesized that males and females could differentially regulate this community in the root endosphere and rhizosphere.

## Materials and methods

### Experimental design and sample collection

Soils were collected from three regions, including Xuanhua County, Hebei Province (40º25′N, 115º02′E), Fushun, Liaoning Province (41°51′N, 124°54′E) and Tianjin (39°40′N, 117°12′E) in China. All the soils from each region were homogeneously mixed and air-dried at Hangzhou Normal University, Hangzhou, China (29°38′N, 119°54′E). The *P. cathayana* cuttings were collected from Qinghai Province (35°56′N, 101°35′E, 2,450 m) and planted in a garden in Hangzhou Normal University. One-year-old cuttings of male and female *P. cathayana* individuals (one cutting per pot) were planted in plastic pots (33 cm in diameter and 22 cm high) that were filled with the homogeneous soil in early March 2021. Seedlings were randomly selected to initiate the experiment at the beginning of May 2021. The experiment contained six treatments with two sexes (male and female) and three soil regions. Each treatment had four replicates. The experiment was conducted in a greenhouse with temperature that ranged from 21 to 25 °C during the day and from 15 to 18 °C at night at Hangzhou Normal University.

Samples, including rhizosphere soil, fine roots (diameter ≤ 2 mm), phloem, and leaves, were collected in mid-September 2021. The rhizosphere soil was defined as soil that closely adheres to the fine roots. One portion of the soil was immediately stored at -80 ºC to extract the DNA. Another portion of the soil was stored at -20 ºC to measure the contents of ammonium ($NH_4^+$) and nitrate ($NO_3^-$). The final portion of the soil samples was air-dried to test the pH and the amounts of total nitrogen (TN), total phosphorus (TP), total potassium (TK), calcium ions ($Ca^{2+}$) and magnesium ions ($Mg^{2+}$). One portion of the fine roots and leaves was washed with sterile $H_2O$ and immersed in 70% ethanol for 5 min. The samples were treated with 5.25% sodium hypochlorite (NaClO) for 5 min and then with 70% ethanol for 30 s. All the surface-sterilized samples were washed with sterile $H_2O$ and stored at -80 ºC until the DNA was extracted. Another portion of the fine roots and leaves was stored at -80ºC to determine the amounts of total phenolics, condensed tannins, and phytohormones, including jasmonic acid (JA), salicylic acid (SA) and indoleacetic acid (IAA). The fresh phloem was stored at -80 ºC after surface sterilization.

### Soil and plant traits

Fresh soil samples were extracted using 50 mL of 2 *M* KCl, and the extracts were used to measure the contents of $NH_4^+$ and $NO_3^-$(Guo et al. 2019). The soil pH was determined from a 1:2.5 ratio of soil: water (v/v). The TN in the soil was determined using the Kjeldahl method. The TP was determined by molybdenum blue colorimetry. The contents of $Mg^{2+}$ and $Ca^{2+}$ were measured from extracts of 1 *M* ammonium acetate (Flannery and Markus 1971). The amount of total K in the soil was determined as described by Singh and Goulding (1997).

The concentrations of JA, SA and IAA were determined using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Briefly, 0.5 g of plant sample was ground into fine powder with liquid nitrogen, and 0.1 mL of 1 ug $mL^{-1}$ standards of JA, SA, and IAA were added (Sigma-Aldrich, St. Louis, MO, USA). The samples were extracted with a 99:1 ratio of methanol: formic acid (v/v) at 4 ºC. The samples were dried using a stream of nitrogen and then dissolved in 2 mL of 10% methanol. A Waters Oasis HLB Column (3 cc/60 mg, Supelco, Sigma-Aldrich) was used to determine the concentrations of phytohormones. The concentration of condensed tannins was determined at 760 nm using the Folin-Denis method. The concentration of total phenolics was measured as described by Guo et al. (2022).

## DNA extraction and amplification

The microbial DNA from the rhizosphere soil was extracted using a PowerSoil DNA Kit (Qiagen, Hilden, Germany), and DNA from the roots, phloem, and leaves was extracted using a FastDNA SPIN Kit for soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The final concentration of DNA and its purification were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the DNA quality was checked by 1% agarose gel electrophoresis.

To amplify the amount of bacterial 16S rRNA in the rhizosphere, the primers 338F (5'-ACTCCT ACGGGAGGCAGCAG-3') and 806R (5'-GGA CTACHVGGGTWTCTAAT-3') (Caporaso et al. 2011) that target the V3-V4 region were utilized for PCR using a thermocycler PCR system (GeneAmp 9700 Fast Thermal Cycler, Thermo Fisher Scientific). A two-round amplification process was used to amplify the 16 S rRNA amplicon of the bacteria that inhabited the roots, phloem, and leaves. The first round of PCR was performed using the primers 799 F (5'-AACMGGATTAGATACCCKG-3') and 1392R (5'-ACG GGCGGTGTGTRC-3') (Samad et al. 2017). The 799 F (5'-AACMGGATTAGAT ACCCKG-3') and 1193R (5'-ACGTCATCC CCA CCTTCC −3') primers were used to target the V5-V7 region of the bacterial 16 S rRNA gene in the second round of PCR (Wang et al. 2018). Nested PCR was conducted to amplify the 18 S rRNA of the AMF. The primer pairs AML1 (5'-ATCAAC TTTCGATGGTAGGATAGA-3') and AML1 (5'-GAACCCAAACACTTTGGTTTCC-3') were used in the first run. The primer pairs AMV4.5NF (5'-AAGCTCGTAGTTGAATTTCG-3') and AMDGR (5'-CCCAACTATCCCTATTAATCAT-3') were used in the second run in the thermocycler PCR system (GeneAmp 9700). The PCR amplification steps followed: initial denaturation (3 min) at 95 °C, 27 cycles (30 s) at 95 °C, annealing (30 s) at 55 °C, elongation (45 s) at 72 °C, and a final extension (10 min) at 72 °C. The PCR products were recovered using 2% agarose Gel, and purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), then was eluted by Tris-HCl, and was detected by 2% agarose electrophoresis using Quantifluor-ST (Promega, USA).

For each sample, purified amplicons were pooled in equimolar amounts and paired-end sequenced (2×300) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The bioinformatics analysis was conducted by following the "Atacama soil microbiome tutorial" of the QIIME2 docs along with customized program scripts (https://docs.qiime2.org/2019.1/). Briefly, raw data FASTQ files were imported into the format that could be operated by the QIIME2 system using the QIIME tools import program. Demultiplexed sequences from each sample were quality filtered and trimmed, de-noised, merged, and the chimeric sequences were then identified and removed using the QIIME2 dada2 plugin to obtain the feature table of amplicon sequence variants (ASVs) or operational taxonomic units (OTUs) (Callahan et al. 2016). The QIIME2 feature-classifier plugin was then used to align the ASV sequences of all the bacteria to a pre-trained GREENGENES 13_8 99% database to generate the taxonomy table. The maarjam20220506/AM species database was used to obtain taxonomic information of the AMF. Any contaminating mitochondrial and chloroplast sequences were filtered using the QIIME2 feature-table plugin. The raw sequencing data were submitted to NCBI: PRJNA982688 (AMF) and PRJNA982680 (bacteria).

## Statistical analysis

The chemical traits of rhizosphere soil and the Shannon diversity of the microbial communities were analyzed by a two-way analysis of variance (ANOVA) to test the site, sex, and their interactive effects, and post-hoc comparisons by Tukey's tests were used to evaluate differences among the treatments. Tukey's tests were also used to evaluate the concentrations of defensive compounds among the different treatments. After that, an independent *t*-test was used to test for significant differences between males and females. The beta-diversity of microbial communities was assessed using a Principle Coordinate Analysis (PCoA) based on Bray-Curtis distances, and permutational multivariate analysis of variance (PERMANOVA) was utilized to test the site, sex, and their interactive effects using the "adonis" function in the vegan package in R (Oksanen et al. 2012). The linear discriminant analysis effect size (LEfSe) with logarithmic LDA > 2 (Wilcoxon $P < 0.05$) was applied to

identify the biomarker taxa at each treatment (https://huttenhower.sph.harvard.edu/galaxy/) (Segata et al. 2011). A Venn diagram was used to visualize intersecting sets of OTUs or ASVs at each treatment (Conway et al. 2017). We defined the dominant taxa that OTUs identified as at least 75% in samples at each plant compartment across three sites and with a relative abundance ≥ 0.1%. 'ggtree' was then used to visualize these dominant taxa (Yu et al. 2017). The molecular ecological networks of each plant compartment were constructed to explore the role of sex in influencing the network traits on the MENAP platform (http://ieg4.rccc.ou.edu/MENA/) after removing the OTUs that had a relative abundance less than 0.01% as described by Feng et al. (2022). 'igraph' was used to visualize the networks. An independent *t*-test was used to compare the topology traits of networks between the males and females. 'ggClusterNet' was used to identify the hub nodes among each bacterial or AMF network (Wen et al. 2022). The dominant taxa, biomarkers, and hub nodes were defined as potential keystone taxa (Xiong et al. 2021). Relationships between the dominant bacterial OTUs with corresponding defensive traits of the hosts, such as condensed tannins, total phenolics, and hormones, were tested. Since males and females share the same dominant OTUs, we also pooled the data of all the plants to test the shared dominant OTUs with plant defensive traits. For AMF taxa, the relationships were tested between ASVs (top 10 ranking in relative abundance) and plant defensive traits. The packages 'psych' and 'ComplexHeatmap' were used (Revelle 2017; Gu 2022).

## Results

The three sites differed in the chemical traits, (Table S1). The sex imposed a substantial interactive effect on these traits, particularly on the concentration of $NH_4^+$. The concentration of $NH_4^+$ in the female rhizosphere was significantly lower than that in the male rhizosphere in the soils from Liaoning and Tianjin (Table S1).

At the phylum level, Proteobacteria, Actinobacteria, and Firmicutes comprised the primary components of the bacterial community in each compartment (Fig. S1). *Delftia* was the primary genus of bacterial endophyte of the leaves, phloem, and roots at each site (Fig. 1). *Streptomyces* was relatively more abundant within the female roots than the male roots at each site (Fig. 1c). The alpha-diversity was significantly higher in the female phloem than in that of the male (Fig. 1e, f). Sex was the primary factor that affected the beta-diversity of the bacterial community in the phloem (Fig. S2). The *Glomeraceae* dominated the AMF community in both the roots and rhizosphere (Fig. S3). The sex factor significantly affected the alpha-diversity of AMF in the roots but not the rhizosphere (Fig. S3).

Male and female *P. cathayana* plants harbored large numbers of unique bacterial OTUs at each plant compartment from the leaf to rhizosphere soil (Fig. S4). For example, the relative abundance of unique OTUs that were identified from Proteobacteria, Actinobacteria, and Firmicutes in the female phloem was higher than that of the male at the Liaoning site. Similarly, a substantial proportion of unique ASVs were identified from the rhizospheres and roots of male or females, respectively (Fig. 2a–f). Most of the unique ASVs were identified from the Glomeraceae and were highly abundant (Fig. 2h, i). The rate of AMF infection of the male roots was significantly higher than that of the female roots in the soil from Tianjin (Fig. 2g). The same or unique dominant endophytes that differed in their relative abundance at each of the three sites were clustered in the Actinobacteria and Proteobacteria in the phylogenetic tree (Fig. 3). In particular, the females harbored more unique dominant OTUs from Actinobacteria and Proteobacteria than the males in the rhizosphere and phloem, respectively. In addition, a dominant OTU that belonged to *Nitrospirae* and a dominant OTU from *Gemmatimonadetes* only occurred in the roots and phloem, respectively (Fig. 3). However, there were more unique dominant OTUs from Proteobacteria in the roots and leaves of males than females. The LEfSe showed that from the rhizosphere to leaves, the males and females harbored different bacterial biomarkers across the three sites (Fig. S5). Similarly, the males and females harbored different AMF biomarkers in the rhizosphere and roots, respectively (Fig. S6).

At each plant compartment, the males and females developed different co-occurrence networks of bacterial and AMF communities (Figs. 4 and 5). In particular, the clustering coefficient, node degree, and negative edge of the bacterial network topological features at the node level were significantly higher

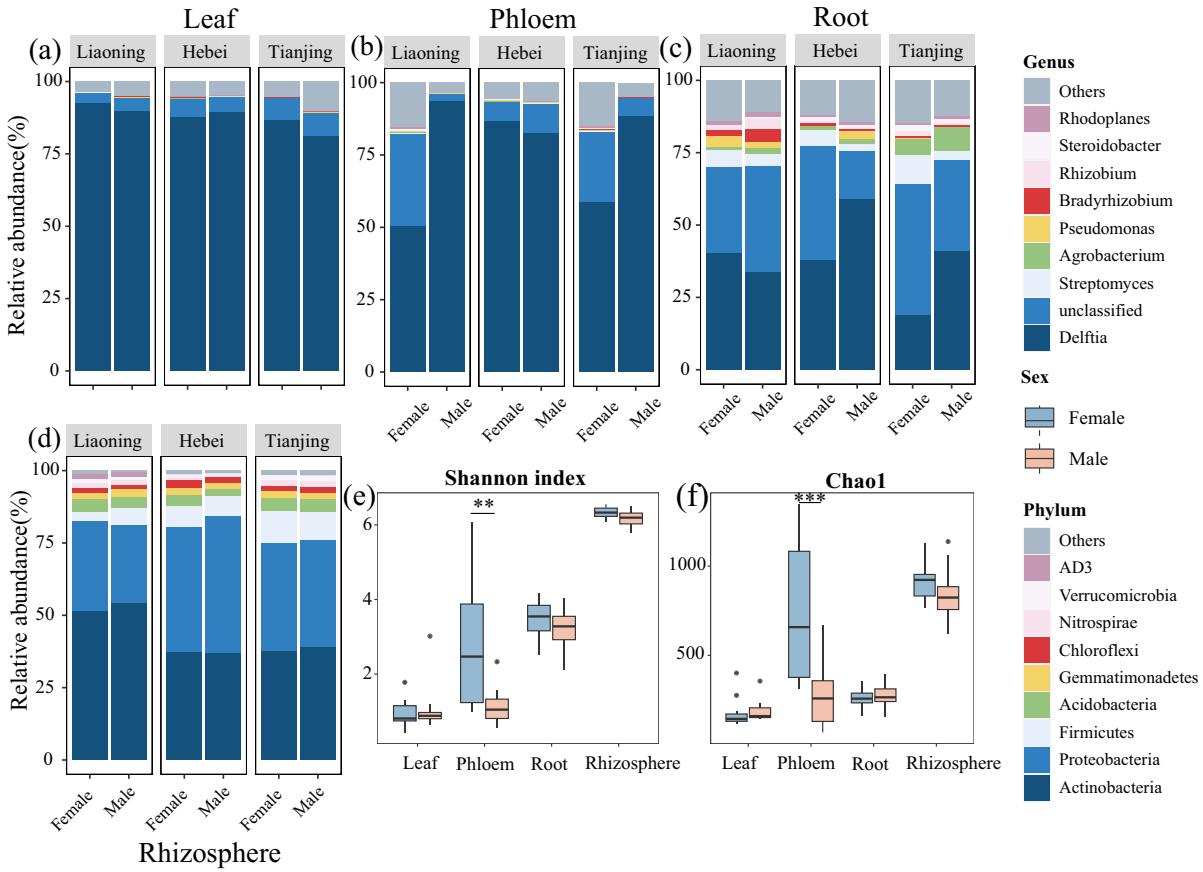


**Fig. 1** The relative abundance of the bacterial communities (**a**-**d**) and the alpha-diversity along the plant-soil continuum between the male and female plants (**e**-**f**). An independent *t*-test was used to compare significant differences of alpha-diversity between the males and females in different plant compartments ($P < 0.05$). *$0.01 \leq P < 0.05$. **$0.001 \leq P < 0.01$. ***$P < 0.001$

in the male leaves than in the female ones (Fig. 4). The node betweenness, positive edge, node degree, and negative edge of the phloem networks were significantly higher in the females than in the males, whereas the node eigenvector centrality scores were lower in the females than the males (Fig. 4). The clustering coefficient, node betweenness, and node degree of the rhizosphere were significantly higher in the females than in the males, whereas the positive and negative edges were lower in the females than in the males (Fig. 4). In each compartment, the composition of hub species in the networks differed substantially between the males and females (Fig. S7). The clustering coefficient, node eigenvector centrality scores, node degree, and negative edge of the AMF community in the male roots were significantly higher than those in the female (Fig. 5). The composition of hub species in the AMF networks differed substantially between males and females in the root and rhizosphere soil, respectively (Fig. S8).

The concentrations of IAA, total phenolics, and condensed tannins in the male leaves tended to be higher than those in the female leaves at each site (Fig. 6). By combining these data, the IAA, total phenolics, and condensed tannins in the male leaves were significantly higher, whereas the concentration of SA was significantly lower when compared with the female leaves (Fig. 6). The concentrations of IAA, JA, and SA in the male roots were significantly higher than those in the female roots (Fig. 6).

The concentrations of defensive compounds that were studied were more significantly related to the relative abundance of dominant OTUs in the root endophytes compared with the leaf endophytes for both males and

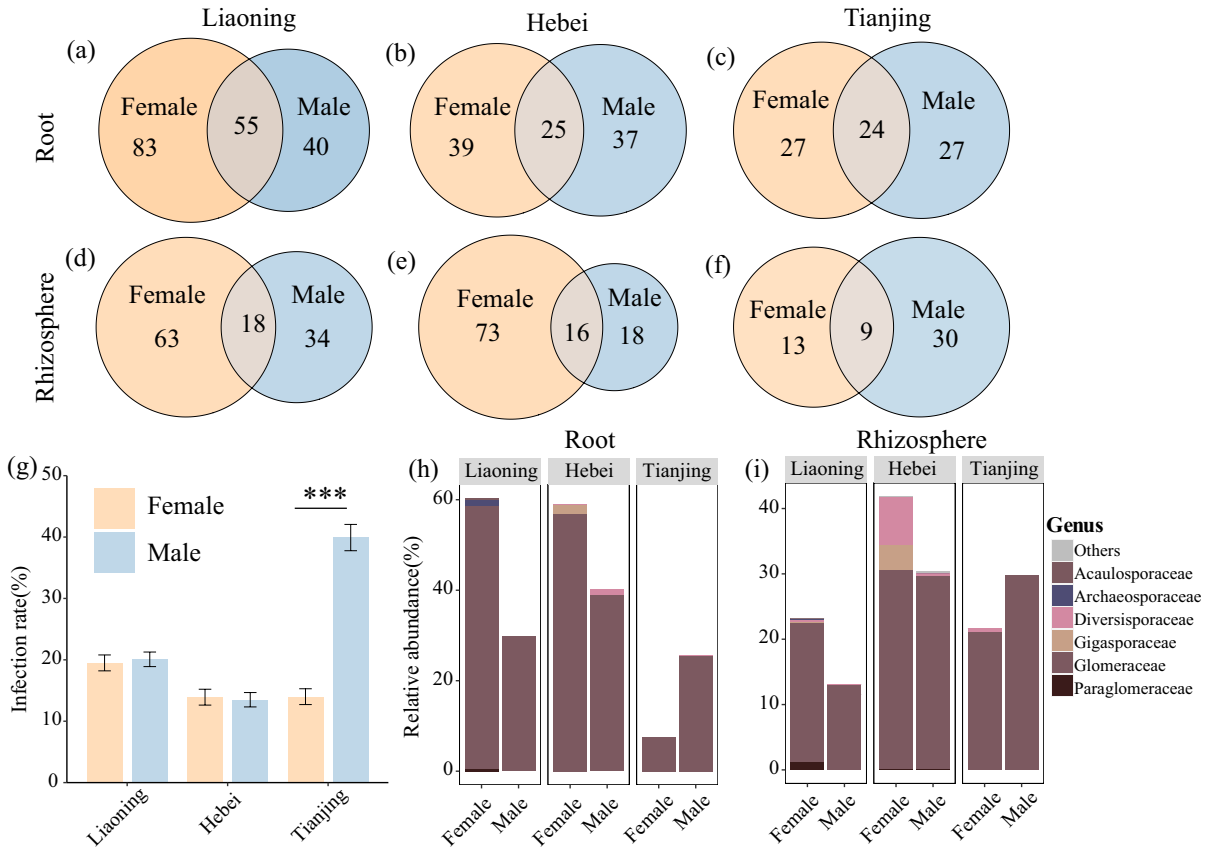


**Fig. 2** The traits of AMF community and mycorrhizal colonization between the males and females. **a-f** The shared and unique ASVs of the AMF communities between the males and females across different sites. **g** The AMF mycorrhizal colonization between the males and females at each site. An independent *t*-test was used to compare significant differences ($P<0.05$). *$0.01\leq P<0.05$. **$0.001\leq P<0.01$. ***$P<0.001$. **h** and **i** The relative abundance of unique ASVs between male and female at each site. AMF, arbuscular mycorrhizal fungi; ASVs, amplicon sequence variants

females (Fig. 7). For bacteria that inhabited the roots, the concentration of total phenolics significantly correlated with the relative abundances of dominant OTUs (bac_10, bac_13, bac_37 and bac_3) in the males ($P=0.04$, $r$ = -0.57; $P=0.007$, $r=0.73$; $P=0.03$, $r=0.73$; $P=0.03$, $r=0.49$) (Fig. 7d). For the shared dominant OTUs between males and females, the condensed tannins positively associated with the relative abundances of bac_7, bac_6 and bac_19 in the root endophytes ($P=0.023$, $r=0.50$; $P=0.04$, $r=0.45$; $P=0.03$, $r=0.49$) (Fig. 7f). The concentration of total phenolics positively correlated with the abundances of bac_16, bac_3, bac_13 and bac_7 ($P=0.04$, $r=0.47$; $P=0.02$, $r=0.47$; $P=0.04$, $r=0.48$; $P=0.03$, $r=0.44$) but negatively correlated with those of bac_1 and bac_10 ($P=0.02$, $r$ = -0.54; $P=0.0004$, $r$ = -0.55) (Fig. 7f). JA had no significant relationships with the shared dominant OTUs, and SA only negatively associated with the abundance of bac_15 ($P=0.008$, $r$ = -0.49) (Fig. 7e, f). The concentration of tannins was significantly associated with the relative abundances of ASV43 and ASV66 in the female roots but had no significant associations with ASVs in the male roots (Fig. S9). The concentrations of JA and SA positively correlated with the abundances of ASV1 and ASV7 but negative correlated with ASV66 in the female roots (Fig. S9).

## Discussion

Many studies have demonstrated that different plant species or genotypes exhibited host selection in the assembly of microbiota in different environments

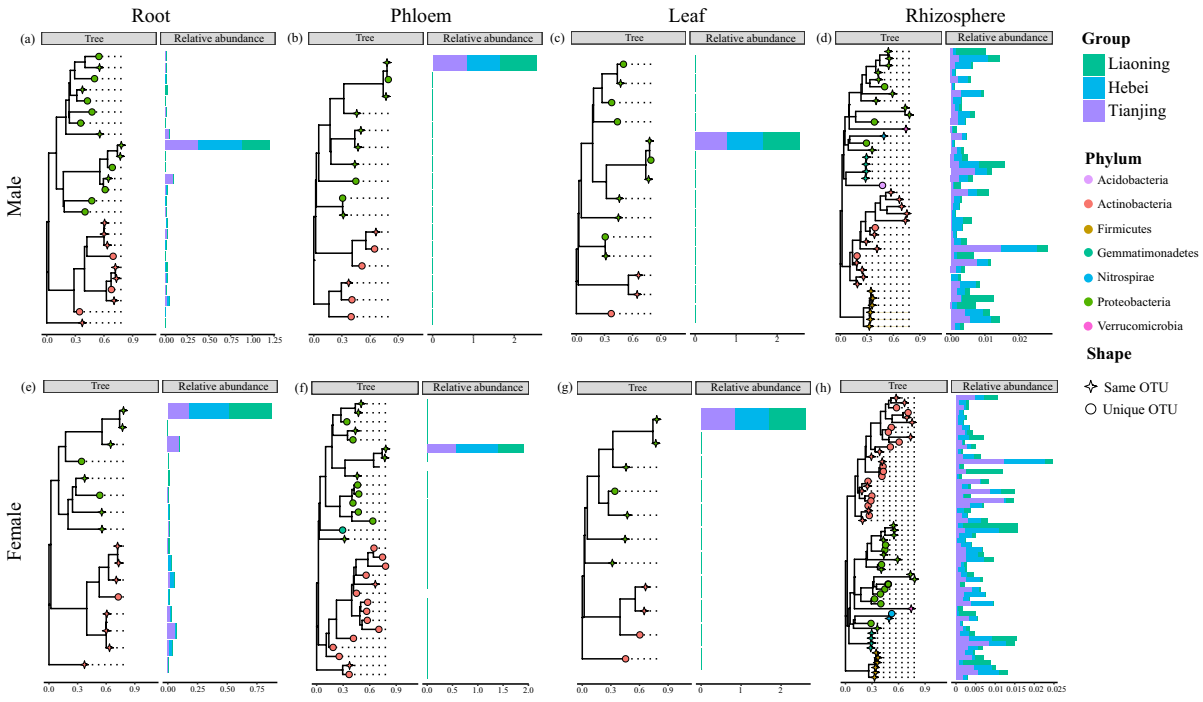


**Fig. 3** The composition and relative abundance of dominant OTUs between the males and females along the plant-soil continuum. The stars and cycles represent shared and unique dominant OTUs between the males and females, respectively. OTUs, operational taxonomic units

(Wagner et al. 2016; Xiong et al. 2021, Guo et al. 2022). Our initial goal was to explore the role of sex in the regulation or selection of sex-specific microbial communities. This study clearly revealed that sex was a crucial factor in the regulation of plant microbiota assembly along the soil-plant continuum, and the selective pressure was much stronger in the endosphere than in the rhizosphere. This study also demonstrated that the composition and structure of bacteria and the AMF communities in endosphere differed in response to changes in the plant defense traits between the male and female *P. cathayana* plants.

## Bacterial communities in the rhizosphere and root endosphere

Plants impose strong control or selection in the structure and function of microbiota in the rhizosphere (Henneron et al. 2020; Henry et al. 2021; Liu et al. 2022). One the one hand, they do this by altering soil chemical traits (Zhalnina et al. 2018). A field study found that male and female Euphrates poplar (*P. euphratica*) plants significantly affected the soil chemical traits, such as TN, soil water, $NH_4^+$, and $Ca^{2+}$, which affected the bacterial and fungal communities of the rhizosphere (Guo et al. 2021). This study also found that the sex significantly affected the soil chemical traits. For example, the concentration of $NH_4^+$ in the male rhizosphere tended to be higher than that in the female rhizosphere (Table S1). Guo et al. (2022) found similar results that the contents of TN and $NH_4^+$ were lower in the rhizosphere soil of female *P. cathayana* plants compared with male ones, probably because of the higher demands of *P. cathayana* females for nutrients for growth. Studies revealed that females with higher specific root lengths can explore and absorb more belowground resources than the males under favorable conditions (Xia et al. 2020, 2022). Alternatively, the control or selection of the rhizosphere microbiota largely depends on the root exudates (Zhalnina et al. 2018). A recent study first demonstrated that the profiles of root exudates obviously differed, and their composition and quantity in responding to environmental changes were also clearly different, which resulted in divergent

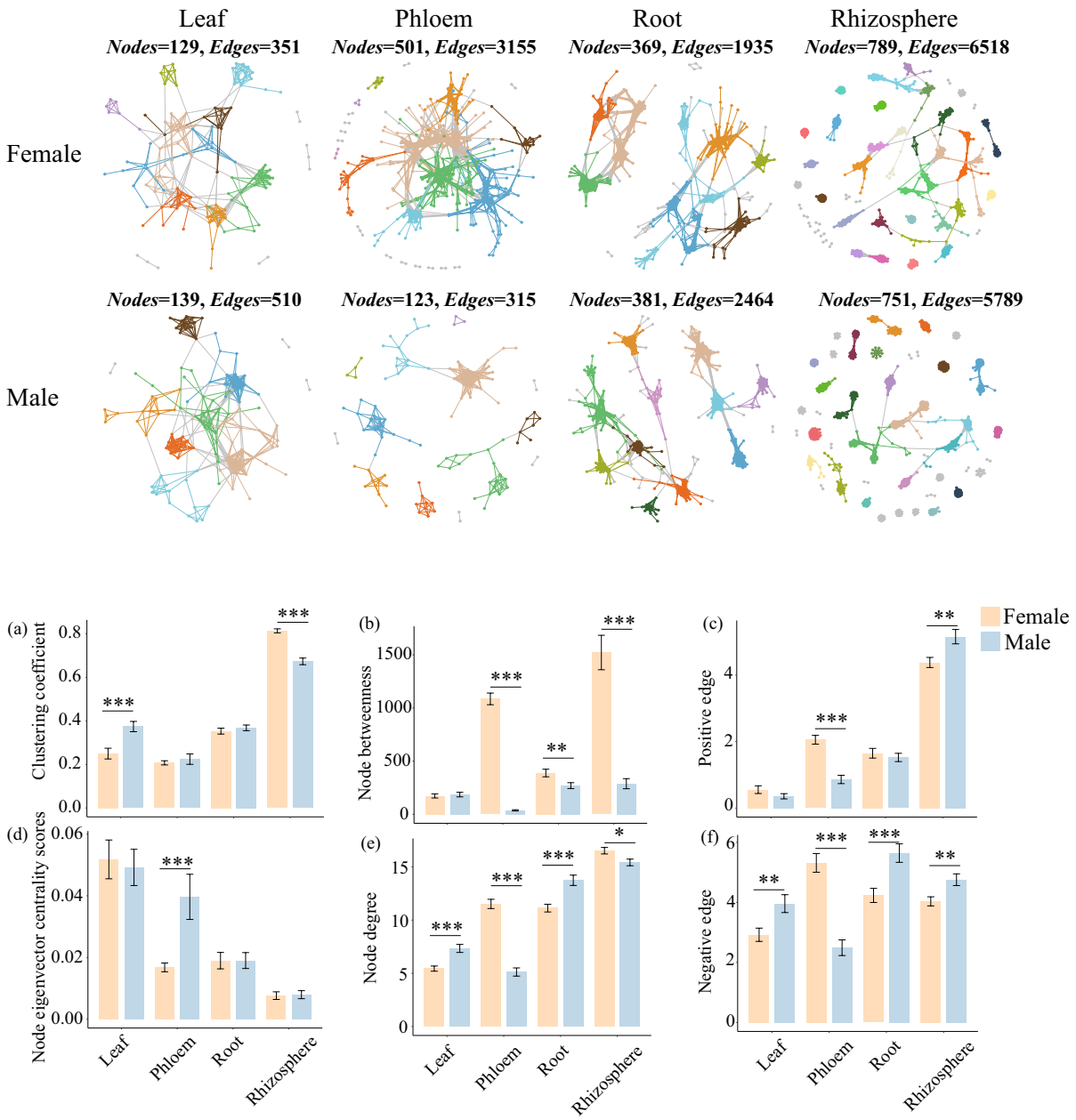


**Fig. 4** The networks of bacterial communities along the plant-soil continuum. The topology traits of these networks were compared between those of males and females in each plant compartment. *$0.01 \leq P < 0.05$. **$0.001 \leq P < 0.01$. ***$P < 0.001$

rhizobacteria between male and female *P. cathayana* plants (He et al. 2022).

The male and female *P. cathayana* plants imposed less effects on diversity, whereas they substantially affected the composition of rhizobacteria, and plants of different sexes harbored large numbers of unique OTUs, biomarkers, and hub species (Figs. S5, S6, and S8). This demonstrated that the root activities of dioecious *P. cathayana*, such as nutrient uptake and root exudates, created divergent microhabitats that provided suitable habitats for different bacterial species to survive and grow. Zhou et al. (2022) found that the metabolic differences in rhizosphere soils caused by root activities

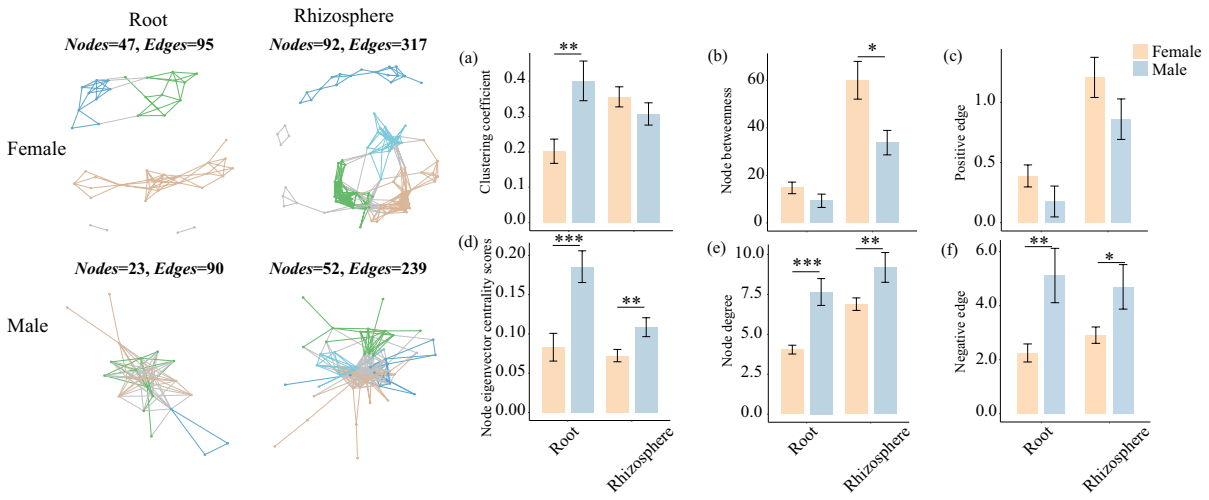


**Fig. 5** The networks of AMF communities in the roots and rhizosphere. The topology traits of these networks were compared between males and females. *$0.01 \le P < 0.05$. **$0.001 \le P < 0.01$. ***$P < 0.001$. AMF, arbuscular mycorrhizal fungi

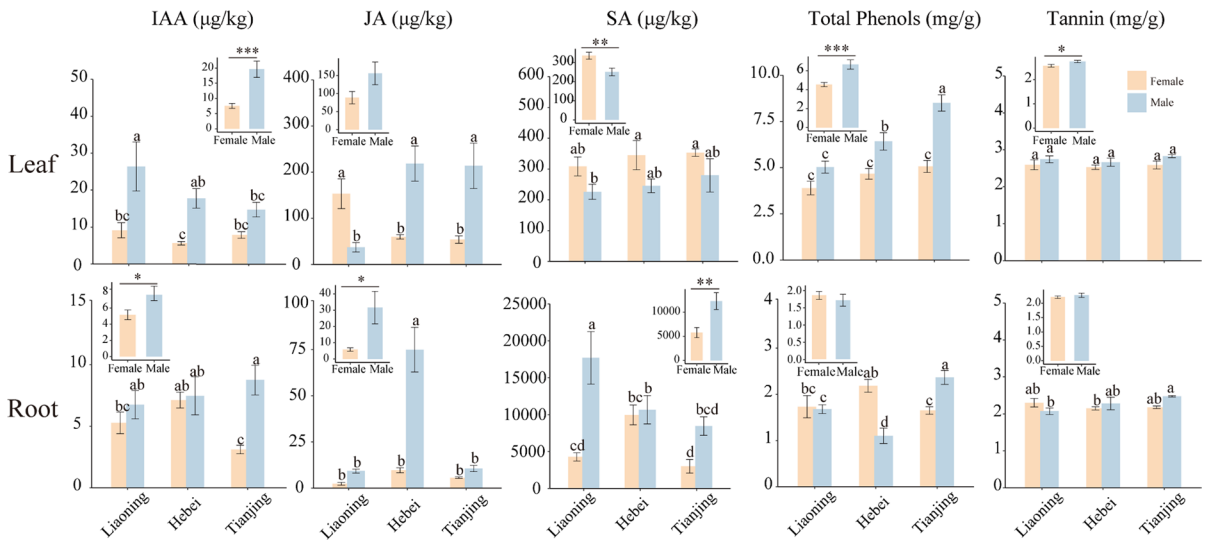


**Fig. 6** The concentrations of condensed tannins, total phenolics, jasmonic acid (JA), salicylic acid (SA) and indoleacetic acid (IAA) in the leaves and roots. The significant differences between males and females were first compared at each site. The data were then pooled to compare the differences between sexes in these defensive traits (the embedded figures). *$0.01 \le P < 0.05$. **$0.001 \le P < 0.01$. ***$P < 0.001$

drove different microbiota assembly between male and female papaya plants (*Carica papaya* L.). Field studies have shown that the divergent rhizobacterial community could affect the processes of nitrogen (N) or phosphorus (P) cycling (Guo et al. 2021; Xia et al. 2022). For example, female *P. euphratica* plants that required high nutrients and grew in an environment with relatively higher amounts of soil water harbored more bacteria that were associated with N cycling (Guo et al. 2021), which is probably

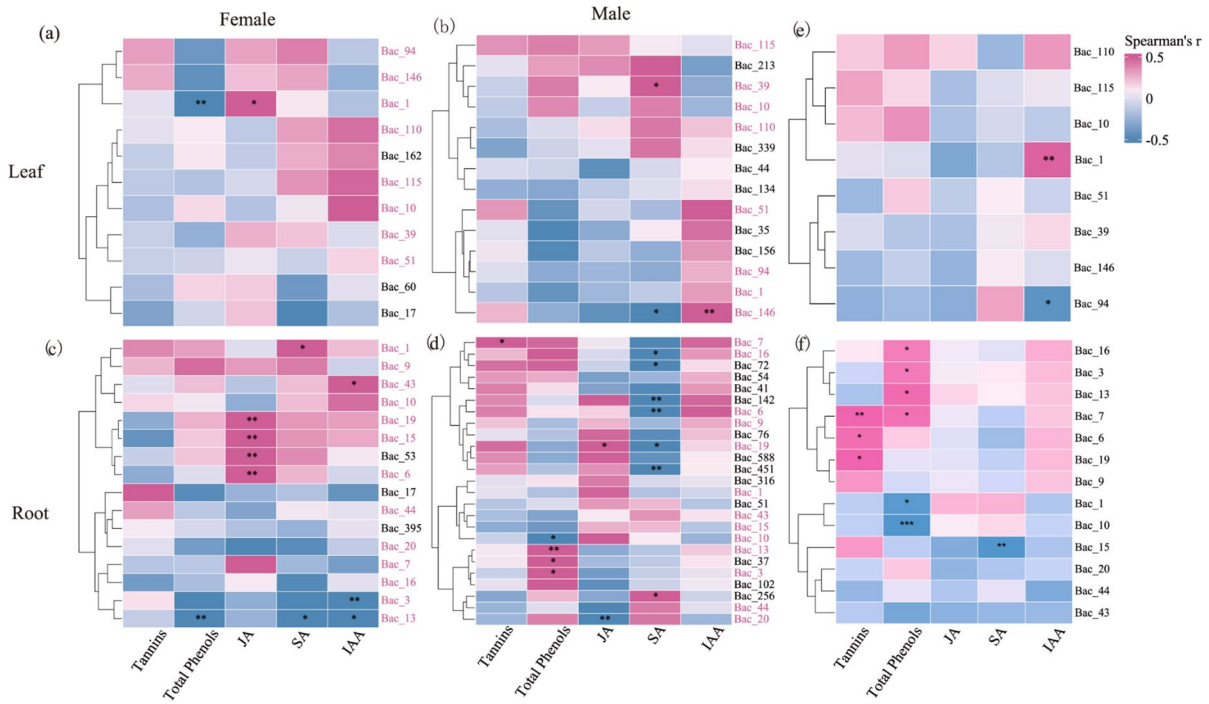


**Fig. 7** The relationships of dominant OTUs with host defensive traits. **a** and **b** The relationships between the dominant OTUs in female and male leaves with defensive traits. **c** and **d** The relationships between the dominant OTUs in female and male roots with defensive traits. The OTU numbers in pink represent the shared dominant OTUs between males and females in leaves (**a** and **b**) and in roots (**c** and **d**), respectively. **e** and **f** The relationships of shared dominant OTUs with defensive traits. We renamed the OTU number (see Table S2). IAA, indole acetic acid; JA, jasmonic acid; OTU, operational taxonomic unit; SA, salicylic acid

a reason that the content of $NH_4^+$ could be affected (Table S1).

## AMF communities in the rhizosphere and root endosphere

The germination of spores and the directional growth of AMF to the host depend on the release of diverse compounds, such as flavonoids, from the roots, and the AMF species cannot grow without the plant host (Pei et al. 2020). Recent studies have demonstrated that the AMF biomass was higher in the rhizosphere of males than females when they grew in soil conditions that were limited in P or water (Xia et al. 2020, 2022). Therefore, it was hypothesized that hosts that release different compounds from the roots probably support different AMF species to survive and grow in the soil. This study clearly demonstrated that males and females harbored large numbers of unique ASVs, biomarkers, and hub species of the AMF community in the rhizosphere (Figs. 2, S7, and S9). These divergent AMF species largely contributed to construct different networks that resulted in a network of male rhizospheres was much closer and more connected than those of the female (Fig. 5). A more complicated AMF network is probably more resistant to environmental changes, which could be an important reason to explain why the arbuscular mycorrhizal hyphal biomass was reduced less in the male *P. euphratica* rhizosphere than in female one when there was a decrease in soil water (Xia et al. 2022). Male poplars have been reported to take up higher amounts of N and P under infertile conditions (Zhang et al. 2014). For example, the males could do this by increasing the availability of soil P compared with those of the females (Xia et al. 2020, 2022). It is hypothesized that the male plants establish more stable symbiotic relationships than the females during environmental changes, but more evidence is needed.

Vega-Frutis et al. (2015) reported that regardless of their phylogenetic relationships, the female plants have a higher frequency of root AMF colonization compared with the male plants. In contrast, Xia et al. (2020) found a higher rate of AMF colonization of male *P. cathayana* roots than those of females in soil that was limited in P. This study only found a significantly higher AMF colonization of males than females in infertile soil collected from Tianjin (Fig. 2g). It suggested that there is a complex interaction between roots and colonization by AMF. However, the composition and abundance of the AMF species that inhabited the roots differed greatly (Figs. 2, S7, and S9) and was affected by defensive compounds (Fig. 7). The significant difference in networks also demonstrated a sexual role in the regulation of microbe-microbe interactions (Fig. 5). The biomarkers and hub species are always regarded as potential keystone species that play crucial functions for their hosts (Agler et al. 2016; Xiong et al. 2021). For example, AMF can induce a more active antioxidant system (Li et al. 2015) and P uptake (Xia et al. 2020) in *P. cathayana* males compared with females, which may be the result of sex selection on the growth and colonization of AMF species. The strong sexual control in AMF species implies that the survival and growth of AMF have to adapt to different cellular chemical environments before successfully establishing mutualistic symbioses.

## Bacterial assembly within plant internal tissues

Except for the rhizobacteria and AMF that inhabit the rhizosphere, bacterial species that colonize internal plant tissues also provide a number of life-support functions for their hosts (Levy et al. 2018; Zhu et al. 2023). Plant hosts impose much stronger selective pressure, and bacterial species must be equipped with distinct traits that allow them to overcome physicochemical barriers and adapt to the metabolic conditions of the endosphere. This study revealed stronger sex effects on the composition, particularly dominant and hub species, diversity, and network structure of the bacterial community in the phloem, which demonstrated a different selection pressure imposed by sex on species selection and microbe-microbe interactions. Females supported a more diverse and complicated bacterial community in their phloem than males (Figs. 1 and 4). In comparison with the males, the thinner cell walls and enhanced carbohydrates of the long-distance transport system of females (Wu et al. 2021; Zhao et al. 2023) may allow more bacterial species to colonize and provide carbon-rich conditions for their growth (Yang et al. 2016).

It is clear that males and females also had significant effects on the composition and structure of the bacterial communities within the roots and leaves. Guo et al. (2022) found similar results on sex regulation on the microbiota assembly in the leaves and roots when the plants grew at different availabilities of N. For example, this study also found that *Streptomyces* was relatively more abundant within the female roots than in the male roots (Fig. 1c). One of the goals of this study was to investigate the roles of defense compounds and phytohormones in regulating a sexually different endophytic community. We found that most of the defensive traits had non-significant relationships with the dominant OTUs in the leaves, whereas they strongly connected with many dominant OTUs that inhabited the roots (Fig. 6). It is interesting to show that the many shared OTUs between males and females were mostly related to the contents of condensed tannins and total phenolics but not those of the hormones. Hormones are reported as go-betweens in plant microbiota assembly that can contribute to microbial diversity in the endosphere (Eichmann et al. 2021). However, Veach et al. (2019) suggested that plant defense strategies partially drive colonization and the assembly of taxon-specific microorganisms. One reason is that these endophytes probably cannot cause a large variation in hormones of the host like those of pathogens during their colonization processes. Another reason is that these species are probably not sensitive to the changes in hormones. In contrast, bacteria have to adapt to high concentrations of defensive compounds, such as tannins and phenolics, in the endosphere, which affects the bacterial community (Guo et al. 2022).

The presence of differences in the plant microbiota in male and female plants emphasizes the crucial role of sex in dioecious plants that we have barely started to discern. This study clearly demonstrated that the plant sex imposed strong pressure in the selection of different bacterial and AMF species and then constructed different networks. The microbiota assembly is a successional, multistep process and depends on an interactive connection among the microbes and

host traits. Different host characters between the male and female plants shape and modulate sex-specific plant microbiota, and in turn, affect the plant fitness of dioecious species to adapt to a changing climate. Plant microbiota have been referred to as the extended or secondary genome of plants during plant evolution, because they can provide additional genes to influence host phenotypes. The sexual differences in plant microbiota might provide external functions for plants under evolutionary pressure. Therefore, more attention is required to explore the connections between the plant microbiota and dioecious species.

**Acknowledgements** This work was supported by the Natural Science Foundation of China (31,800,508).

**Author contributions** Yuanjing Zhu, Tingting Dong, Fangyuan Sun and Yuxin Xiao collected the data; Yuanjing Zhu analyzed the data and prepared figures; Qingxue Guo conceived the ideas, designed methodology and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

**Data availability** The raw sequencing data were submitted to NCBI: PRJNA982688 (AMF) and PRJNA982680 (bacteria). The datasets analyzed during this study are available from the corresponding author upon reasonable request.

**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

Agler MT, Ruhe J, Kroll S, Morhenn C, Kim ST, Weigel D, Kemen EM (2016) Microbial hub taxa link host and abiotic factors to plant microbiome variation. Plos Biol 14:e1002352. https://doi.org/10.1371/journal.pbio.1002352

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583. https://doi.org/10.1038/nmeth.3869

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. P Nat Acad Sci US A108:4516–4522. https://doi.org/10.1073/pnas.1000080107

Charlesworth D (2002) Plant sex determination and sex chromosomes. Heredity 88:94–101

Conway JR, Lex A, Gehlenborg N (2017) UpSetR: an R package for the visualization of intersecting sets and their properties. Bioinformatics 33:2938–2940. https://doi.org/10.1093/bioinformatics/btx364

Cordovez V, Dini-Andreote F, Carrión VJ, Raaijmakers JM (2019) Ecology and evolution of plant microbiomes. Annu Rev Microbiol 73:69–88. https://doi.org/10.1146/annurev-micro-090817-062524

Eichmann R, Richards L, Schafer P (2021) Hormones as go-betweens in plant microbiome assembly. Plant J 105:518–541. https://doi.org/10.1111/tpj.15135

Eppley SM, Mercer CA, Haaning C, Graves CB (2009) Sex-specific variation in the interaction between *Distichlis spicata* (Poaceae) and mycorrhizal fungi. Am J Bot 96:1967–1973. https://doi.org/10.3732/ajb.0900076

Feng K, Peng X, Zhang Z, Gu S, He Q, Shen W, Wang Z, Wang D, Hu Q, Li Y, Wang S, Deng Y (2022) iNAP: an integrated network analysis pipeline for microbiome studies. iMeta 1:e13. https://doi.org/10.1002/imt2.13

Flannery RL, Markus DK (1971) Determination of phosphorus, potassium, calcium, and magnesium simultaneously in North Carolina, Ammonium Acetate, and Bray P1 soil extracts by AutoAnalyzer. Instrumental Methods for Analysis of Soils and Plant Tissue. https://doi.org/10.2136/1971.instrumentalmethods.c6

Genre A, Lanfranco L, Perotto S, Bonfante P (2020) Unique and common traits in mycorrhizal symbioses. Nat Rev Microbiol 18:649–660. https://doi.org/10.1038/s41579-020-0402-3

Gu Z (2022) Complex heatmap visualization. iMeta 1: e43. https://doi.org/10.1002/imt2.43

Guo QX, Yan LJ, Korpelainen H, Niinemets Ü, Li CY (2019) Plant-plant interactions and N fertilization shape soil bacterial and fungal communities. Soil Biol Biochem 128:127–138. https://doi.org/10.1016/j.soilbio.2018.10.018

Guo QX, Liu JT, Yu L, Korpelainen H, Li CY (2021) Different sexual impacts of dioecious *Populus euphratica* on microbial communities and nitrogen cycle processes in natural forests. For Ecol Manag 496:119403. https://doi.org/10.1016/j.foreco.2021.119403

Guo QX, Liu L, Liu JT, Korpelainen H, Li CY (2022) Plant sex affects plant-microbiome assemblies of dioecious *Populus cathayana* trees under different soil nitrogen conditions. Microbiome 10:191. https://doi.org/10.1186/s40168-022-01387-9

Guo QX, Zhu YJ, Korpelainen H, Niinemets Ü, Li CY (2023) How does plant sex alter microbiota assembly in dioecious plants? Trends Microbiol 31:894–902. https://doi.org/10.1016/j.tim.2023.03.014

He Y, Zhu ZD, Zhou ZH, Lu T, Kumar A, Xia ZC (2022) Foliar application of lambda-cyhalothrin modulates root exudate profile and the rhizosphere bacteria community of dioecious Populus cathayana. Environ Pollut 313. https://doi.org/10.1016/j.envpol.2022.120123

Henneron L, Kardol P, Wardle DA, Cros C, Fontaine S (2020) Rhizosphere control of soil nitrogen cycling: a key component of plant economic strategies. New Phytol 228:1269–1282. https://doi.org/10.1111/nph.16760

Henry LP, Bruijning M, Forsberg SKG, Ayroles JF (2021) The microbiome extends host evolutionary potential. Nat Commun 12:5141. https://doi.org/10.1038/s41467-021-25315-x

Juvany M, Munne-Bosch S (2015) Sex-related differences in stress tolerance in dioecious plants: a critical appraisal in

a physiological context. J Exp Bot 66:6083–6092. https://doi.org/10.1093/jxb/erv343

Keymer A, Pimprikar P, Wewer V, Huber C, Brands M, Bucerius SL, Delaux PM, Klingl V, Röpenack-Lahaye EV, Wang TL, Eisenreich W, Dörmann P, Parniske M, Gutjahr C (2017) Lipid transfer from plants to arbuscular mycorrhiza fungi. Elife 6:e29107. https://doi.org/10.7554/eLife.29107

Kim YS, Unno T, Kim BY, Park MS (2020) Sex differences in gut microbiota. World J Mens Health 38:48–60. https://doi.org/10.5534/wjmh.190009

Koliada A, Moseiko V, Romanenko M, Lushchak O, Kryzhanovska N, Guryanov V, Vaiserman A (2021) Sex differences in the phylum-level human gut microbiota composition. BMC Microbiol 21. https://doi.org/10.1186/s12866-021-02198-y

Levy A, Gonzalez IS, Mittelviefhaus M, Clingenpeel S, Paredes SH, Miao JM, Wang KR, Devescovi G, Stillman K, Monteiro F, Alvarez BR, Lundberg ADS, Lu TY, Lebeis S, Jin Z, McDonald M, Klein AP, Feltcher ME, Rio TG, Grant SR, Doty SL, Ley RE, Zhao BY, Venturi V, Pelletier DA, Vorholt JA, Tringe SG, Woyke T, Dangl JL (2018) Genomic features of bacterial adaptation to plants. Nat Genet 50:138. https://doi.org/10.1038/s41588-017-0012-9

Li Z, Wu N, Liu T, Chen H, Tang M (2015) Effect of arbuscular mycorrhizal inoculation on water status and photosynthesis of *Populus cathayana* males and females under water stress. Physiol Plant 155:192–204. https://doi.org/10.1111/ppl.12336

Lin T, Tang J, Li S, Li S, Han S, Liu Y, Yang C, Chen G, Chen L, Zhu T (2023) Drought stress mediated differences in phyllosphere microbiome and associated pathogen resistance between male and female poplars. Plant J 115:1100–1113. https://doi.org/10.1111/tpj.16283

Liu JT, Wang XY, Liu L, Wu XF, Xia ZC, Guo QX (2022) Rhizosphere soil bacterial communities and nitrogen cycling affected by deciduous and evergreen tree species. Ecol Evol 12:e9103. https://doi.org/10.1002/ece3.9103

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Sólymos P, Stevens MHH, Wagner H (2012) vegan: Community Ecology Package. Software http://CRAN.R-project.org/package=vegan. Accessed 2012

Pei Y, Siemann E, Tian B, Ding J (2020) Root flavonoids are related to enhanced AMF colonization of an invasive tree. AoB Plants 12:plaa002. https://doi.org/10.1093/aobpla/plaa002

Renner SS (2014) The relative and absolute frequencies of angiosperm sexual systems: dioecy, monoecy, gynodioecy, and an updated online database. Am J Bot 101:1588–1596. https://doi.org/10.3732/ajb.1400196

Revelle WR (2017) Psych: procedures for personality and psychological research. Northwestern University. https://personalityproject.org/r/psych/. Accessed 2017

Samad A, Trognitz F, Compant S, Antonielli L, Sessitsch A (2017) Shared and host-specific microbiome diversity and functioning of grapevine and accompanying weed plants. Environ Microbiol 19:1407–1424. https://doi.org/10.1111/1462-2920.13618

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C (2011) Metagenomic biomarker discovery and explanation. Genome Biol 12:R60. https://doi.org/10.1186/gb-2011-12-6-r60

Singh B, Goulding KWT (1997) Changes with time in the potassium content and phyllosilicates in the soil of the Broadbalk continuous wheat experiment at Rothamsted. Eur J Soil Sci 48:651–659. https://doi.org/10.1111/j.1365-2389.1997.tb00565.x

Song HF, Cai ZY, Liao J, Tang DT, Zhang S (2019) Sexually differential gene expressions in poplar roots in response to nitrogen deficiency. Tree Physiol 39:1614–1629. https://doi.org/10.1093/treephys/tpz057

Song HF, Cai Z, Liao J, Zhang S (2020) Phosphoproteomic and metabolomic analyses reveal sexually differential regulatory mechanisms in poplar to nitrogen deficiency. J Proteome Res 19:1073–1084. https://doi.org/10.1021/acs.jproteome.9b00600

Trivedi P, Leach JE, Tringe SG, Sa T, Singh BK (2021) Plant-microbiome interactions: from community assembly to plant health. Nat Rev Microbiol 19:72–72. https://doi.org/10.1038/s41579-020-00490-8

Veach AM, Morris R, Yip DZ, Yang ZK, Engle NL, Cregger MA, Tschaplinski TJ, Schadt CW (2019) Rhizosphere microbiomes diverge among *Populus trichocarpa* plant-host genotypes and chemotypes, but it depends on soil origin. Microbiome 7:76. https://doi.org/10.1186/s40168-019-0668-8

Vega-Frutis R, Guevara R (2009) Different arbuscular mycorrhizal interactions in male and female plants of wild *Carica papaya* L. Plant Soil 322:165–176. https://doi.org/10.1007/s11104-009-9903-6

Vega-Frutis R, Lopez JC, Flandes C, Guevara R (2015) Have male trees of the tropical rain forest evolved to minimize the interactions with mycorrhizal symbionts? Perspect Plant Ecol Evol Syst 17:444–453. https://doi.org/10.1016/j.ppees.2015.09.004

Wagner MR, Lundberg DS, del Rio TG, Tringe SG, Dangl JL, Mitchell-Olds T (2016) Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Nat Commun 7:12151. https://doi.org/10.1038/ncomms12151

Wang F, Men X, Zhang G, Liang KC, Xin YH, Wang J, Li AJ, Zhang HB, Liu HB, Wu LJ (2018) Assessment of 16S rRNA gene primers for studying bacterial community structure and function of aging flue-cured tobaccos. AMB Express 8. https://doi.org/10.1186/s13568-018-0713-1

Wen T, Xie P, Yang S, Niu G, Liu X, Ding Z, Xue C, Liu YX, Shen Q, Yuan J (2022) ggClusterNet: an R package for microbiome network analysis and modularity-based multiple network layouts. iMeta 1:e32. https://doi.org/10.1002/imt2.32

Wu XY, Liu JT, Meng QQ, Fang SY, Kang JY, Guo QX (2021) Differences in carbon and nitrogen metabolism between male and female *Populus cathayana* in response to deficient nitrogen. Tree Physiol 41:119–133. https://doi.org/10.1093/treephys/tpaa108

Xia ZC, He Y, Yu L, Lv RB, Korpelainen H, Li CY (2020) Sex-specific strategies of phosphorus (P) acquisition in *Populus cathayana* as affected by soil P availability and distribution. New Phytol 225:782–792. https://doi.org/10.1111/nph.16170

Xia ZC, He Y, Zhu ZD, Korpelainen H, Li CY (2022) Covariations and trade-offs of phosphorus (P) acquisition

strategies in dioecious *Populus Euphratica* as affected by soil water availability. Funct Ecol 36:3188–3199. https://doi.org/10.1111/1365-2435.14193

Xiong C, Singh BK, He JZ, Han YL, Li PP, Wan LH, Guo ZH, Liu SY, Wang JT, Wu CF, Ge AH, Zhang LM (2021) Plant developmental stage drives the differentiation in ecological role of the maize microbiome. Microbiome 9:171. https://doi.org/10.1186/s40168-021-01118-6

Yang T, Weisenhorn P, Gilbert JA, Ni YY, Sun RB, Shi Y, Chu HY (2016) Carbon constrains fungal endophyte assemblages along the timberline. Environ Microbiol 18:2455–2469. https://doi.org/10.1111/1462-2920.13153

Yu GC, Smith DK, Zhu HC, Guan Y, Lam TTY (2017) GGTREE: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods Ecol Evol 8:28–36. https://doi.org/10.1111/2041-210x.12628

Zhalnina K, Louie KB, Hao Z, Mansoori N, da Rocha UN, Shi SJ, Cho HJ, Karaoz U, Loque D, Bowen BP, Firestone MK, Northen TR, Brodie EL (2018) Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. Nat Microbiol 3:470–480. https://doi.org/10.1038/s41564-018-0129-3

Zhang S, Jiang H, Zhao HX, Korpelainen H, Li CY (2014) Sexually different physiological responses of Populus cathayana to nitrogen and phosphorus deficiencies. Tree Physiol 34:343–354. https://doi.org/10.1093/treephys/tpu025

Zhao W, Lin X, Wang Y, Yang Q, Liu M (2023) Nitrogen level induces sex-specific cadmium phloem remobilization and cell wall segregation in *Populus cathayana*. Sci Total Environ 890:164184. https://doi.org/10.1016/j.scitotenv.2023.164184

Zhou Y, Pang Z, Yuan Z, Fallah N, Jia H, Ming R (2022) Sex-based metabolic and microbiota differences in roots and rhizosphere soils of dioecious papaya (Carica papaya L). Front Plant Sci 13:991114. https://doi.org/10.3389/fpls.2022.991114

Zhu YG, Peng J, Chen C, Xiong C, Li S, Ge A, Wang E, Liesack W (2023) Harnessing biological nitrogen fixation in plant leaves. Trends Plant Sci. https://doi.org/10.1016/j.tplants.2023.05.009