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Effects of pesticide application and plant sexual identity on leaf physiological traits and phyllosphere bacterial communities

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Abstract

Pesticides are widely used to enhance food production on a global scale. However, little information is available on the effects of pesticide application on leaf physiology and phyllosphere bacterial communities of dioecious plants. Therefore, this study aimed to assess the impact of λ-cyhalothrin, a broad-spectrum pesticide, on leaf physiology and phyllosphere bacterial communities in the dioecious *Populus cathayana*. Physiological leaf traits such as photosynthetic apparatus (net photosynthetic rate (P_n), stomatal conductance (g_s) and transpiration (E)) of males were significantly higher than those of females, independent of pesticide use. In contrast, pesticide application significantly reduced the photosynthetic apparatus for both sexes, and the reduction was greater in males relative to females. Also, pesticide application significantly increased peroxidase (POD) activity and malondialdehyde (MDA) content and maintained superoxide dismutase (SOD) activity and total chlorophyll content in leaves of males. The phyllosphere bacteria showed some conserved characteristics, in which, Simpson and Shannon diversity indices were not affected by sex or pesticide application. Phyllosphere bacterial community composition differed between females and males, indicating that intrinsic sex significantly shapes the phyllosphere bacterial community. However, pesticide application significantly increased the relative abundance of Actinobacteria but reduced the relative abundance of Proteobacteria. Principal component analysis showed associations between leaf physiology and specific bacterial taxa. For instance, Proteobacteria negatively correlated with leaf SOD activity and MDA content, while Actinobacteria showed an opposite pattern. Our study highlights sex-specific phyllosphere bacterial community composition and leaf physiological traits in dioecious plants.

Keywords dioecious plants, λ-cyhalothrin, leaf physiology, phyllosphere bacterial community, sex-specific responses

杀虫剂施用和植物性别对叶片生理特性和叶际细菌群落的影响

摘要：在全球范围内，杀虫剂被广泛应用于提高粮食产量。然而，关于施用杀虫剂对雌雄异株植物的叶片生理和叶际细菌群落影响的信息很少。因此，本研究旨在评估高效氯氟氰菊酯(一种广谱杀虫剂)对雌雄异株青杨(*Populus cathayana*)叶片生理和叶际细菌群落的影响。雄性青杨叶片的光合参数(净光合速率(P_n)、气孔导度(g_s)和蒸腾作用(E))显著高于雌性，且与杀虫剂的施用无关。但施用杀虫剂后，雌性和雄性的光合相关参数均显著降低。相对于雌性，雄性的降低幅度更大。同时，施用杀虫剂导致雄性叶片的过氧化物酶(POD)活性和丙二醛(MDA)含量显著增加，而超氧化物歧化酶(SOD)活性和总叶绿素含量保持不变。叶际细菌群落表现出一定的保守特性，如辛普森(Simpson)和香农(Shannon)多样性指数不受性别和杀虫剂施用的影响。然而，雌性和雄性青杨的叶际细菌群落的组成存在差异，表明内在的性别特性显著塑造了叶际细菌群落。另一方面，杀虫剂的施用显著增加了放线菌门(Actinobacteria)的相对丰度，但却减少了变形菌门(Proteobacteria)的相对丰度。主成分分析表明，叶片生理与特定细菌类群间存在关联。例如，变形菌门(Proteobacteria)与叶片SOD活性和MDA含量呈负相关，而放线菌门(Actinobacteria)则表现出相反的模式。我们的研究强调雌雄异株植物的叶际细菌群落组成和叶片生理特征存在性别特异性。

关键词：雌雄异株植物，高效氯氟氰菊酯，叶片生理，叶际细菌群落，性别特异性响应

INTRODUCTION

Poplars are among the widely distributed and adaptable tree species in the world. Planting poplars have substantial economic, ecological and social benefits (Wu *et al.* 2020a). However, poplars suffer from various pest infestations throughout their lifespan that leads to usage of pesticides to prevent damages (Wang *et al.* 2021). Although pesticides can effectively inhibit insect feeding, their heavy use results in severe environmental impacts, including agricultural non-point source pollution, increased costs, effects on beneficial invertebrates and threats to food safety and quality (Bashir *et al.* 2022; Huang *et al.* 2021). Many studies have focussed on the impact of pesticide application on plant–microbe interactions (Chen *et al.* 2021; Feng *et al.* 2020; Laforest-Lapointe *et al.* 2016; Nettles *et al.* 2016; Qu *et al.* 2021; Zheng *et al.* 2021). Moreover, pesticide application leads to variation in microbial community composition and functioning in soil, rhizosphere and root and seed endophytes (Guo *et al.* 2020; He *et al.* 2022). However, research investigating the impact of pesticide application on microbiome of phyllosphere hierarchy is rare.

The plant phyllosphere is occupied by many microorganisms, including bacteria, fungi, archaea and protists (Berg *et al.* 2016). These microorganisms play an essential role throughout the life cycle of plants and have a positive impact on plant health, growth and productivity (Abadi *et al.* 2021; Van der Heijden *et al.* 2016; Verma *et al.* 2014). They are involved in nitrogen fixation and bioremediation of organic pollutants, provide disease resistance, and therefore, are virtual extension of the plant's immune system (Carrión *et al.* 2019). The bacterial community of plant phyllosphere is steered by specific assembly history factors, including the plant itself and the external environment (Bodenhausen *et al.* 2014; Cernava *et al.* 2019; Lajoie *et al.* 2020). Pesticide application could result in changes in phyllosphere bacterial community composition, and therefore, functioning. Previous studies have shown that the relative abundance of Proteobacteria in both tobacco (*Nicotiana tabacum*) and wheat (*Triticum aestivum*) phyllosphere reduced after pesticide application (Chen *et al.* 2021; Xu *et al.* 2020). In contrast, the relative abundance of Actinobacteria in tea (*Camellia sinesis*) phyllosphere increased after pesticide application (Cernava *et al.* 2019). Differences in leaf functional traits, nutrient content and primary and secondary metabolites among plant types may lead to distinct microbial community composition and likely to respond specifically to identity of applied pesticide (Zhu *et al.* 2022). Therefore, a systematic comparison of the impact of pesticides on phyllosphere bacterial community composition of varying plant types is still lacking. It is not clear whether the response of phyllosphere bacteria to pesticides is consistent across species.

Populus cathayana is a typical dioecious tree. Females and males show sexual dimorphism in morphology, physiology and response to biotic and abiotic stress (Liu *et al.* 2021c; Xia *et al.* 2020a; Zhang *et al.* 2019).

Sex-related differences in secondary sexual dimorphism are derived from reproductive costs (Juvany and Munne-Bosch 2015; Liu *et al.* 2021b; Retuerto *et al.* 2018). Females allocate more resources to reproductive organs and later to fruits and seeds (Randriamanana *et al.* 2014). From our previous studies, it was evident that females and males had differences in leaf metabolic profile where females typically allocate more resources to chemical defense and tolerance to insect herbivory feeding when compared with males (He *et al.* 2021). Meanwhile, females and males showed sexual differences in antioxidant defense systems (superoxide dismutase (SOD) and peroxidase (POD) activity), biochemical characteristics (malondialdehyde (MDA) content) (Zhao *et al.* 2009) and the phyllosphere bacterial community (Liu *et al.* 2021a). However, it is unclear whether application of agrochemicals (i.e. pesticides) would lead to changes in phyllosphere microbial community composition of dioecious plants in a sex-specific manner.

Populus cathayana faces severe pest feeding, and forestry workers often apply pesticides to reduce the economic losses (Shelton *et al.* 2003; Wu *et al.* 2020b). However, we do not yet know the effects of pesticide application on the leaf physiology of females and males and on phyllosphere bacterial communities. Therefore, we set up a pot experiment to compare the changes in leaf photosynthesis, physiology and phyllosphere bacterial community of males and females with and without the application of a broad-spectrum pesticide (λ-cyhalothrin). It has been previously shown that λ-cyhalothrin is effective in pest control against leaf-feeding insects of Notodontidae on poplars (Shi *et al.* 2017). Therefore, we hypothesized (i) sex-specific responses in leaf photosynthetic efficiency and physiological traits exposed to pesticide application, and (ii) the phyllosphere bacterial communities of females and males would respond distinctly to pesticide application. Our research would be helpful to develop a better understanding of the bacterial community of dioecious plants in response to pesticide application and improve our knowledge of the future development of differentiated microbial-based biological monitoring systems to assess the integrity of phyllosphere microbial niches.

MATERIALS AND METHODS

Plant material and soils

Shoot cuttings of *P. cathayana* were obtained from 24 different trees of both sexes, sampled from 6 populations (8 adult trees per population of each sex) from Qinghai Province, China (35°56′ N, 101°35′ E; for more details, see Xia *et al.* 2020a). Cuttings were approximately 18–20 cm in height and 2 cm in diameter with two to four dormant buds. Sandy soils were collected from an experimental site at the Hangzhou Normal University in Zhejiang. Soils were air-dried and sieved (2 mm). The soil properties were as follows: 2.82 g kg^{-1} organic matter content, 0.28 g kg^{-1} total nitrogen (N), 2.62 mg kg^{-1} available phosphorus (P) and 90.65 mg kg^{-1} available potassium (K) and pH 8.64. The experiment was conducted in a glasshouse at the Hangzhou Normal University in Zhejiang, with the temperature at 21–25 °C during the day and 15–18 °C at night, and 12–14 h photoperiod throughout the growth period.

Experimental setup

To assess how dioecious *P. cathayana* respond to pesticide application, a pot experiment was conducted in a glasshouse in a factorial design: two sexes (shoot cuttings from male and female trees) and pesticide application (λ-cyhalothrin, hereafter λ-CY) and control (CK) with six replicates for each treatment combination thereby leading to 24 pots. The λ-CY (5% ME) was purchased from MedChemExpress (MCE®, USA). The stock solution of λ-CY was diluted with deionized water to a concentration of 62.5 mg L^{-1} before each application. This dosage has been considered to be very effective against most leaf-feeding insects and was often used by Chinese forestry workers when applying pesticides (Balanza *et al.* 2021). Each application of pesticide was preceded by sealing the pot surface with plastic wrap to prevent the pesticide from entering the soil. The diluted pesticide was sprayed onto all leaves of females and males *P. cathayana* with a sprinkler, and the pesticide was applied every month for a total of four times through the growing season (30 May, 30 June, 30 July and 30 August 2021), each with 83 mL per plant. For control (CK) pots, similar volume of deionized water was sprayed at each time interval. Harvesting took place 1 day after the last pesticide application (31 August 2021).

Leaf photosynthesis measurements

On the day of harvest, the photosynthetic characteristics were measured fourth fully expanded leaf with the Li-6800 photosynthesis measuring system (Li-Cor, Inc., Lincoln, NE, USA) between 08:00 and 11:30 h. Net photosynthetic rate (P_n, μmol m^{-2} s^{-1}), stomatal conductance (g_s, mol m^{-2} s^{-1}) and transpiration rate (E, mmol m^{-2} s^{-1}) were measured (Supplementary Note S1).

Leaf physiological traits

The SOD and POD activity, MDA content and total chlorophyll concentration were measured as described by Zhao *et al.* (2009) and Zhang *et al.* (2012). SOD activity was quantified by measuring the ability to inhibit photochemical reduction of nitroblue tetrazolium. POD activity was quantified at the absorbance of 436 nm in 2 mL of 100 mM potassium phosphate buffer (pH 6.5) containing 40 mmol L^{-1} guaiacol, 10 mmol L^{-1} H_2O_2 and enzyme extract comprising 100 µg protein (Zhang *et al.* 2012). In addition, MDA content was determined by measuring the thiobarbituric acid-reactive substances and measured spectrophotometrically at 450, 532 and 600 nm (Zhao *et al.* 2009). For chlorophyll concentration, 0.05 g of leaf discs were put into 5 mL centrifuge tubes and extracted with 5 mL of 95% ethanol for 24 h. After centrifugation, the absorbance values were measured at the absorbance of 663 nm for chlorophyll a and 665 nm for chlorophyll b, and the total chlorophyll concentration was calculated as the sum of chlo a and chlo b (Zhao *et al.* 2009).

Phyllosphere bacterial community

Microbial cells were collected from 10 to 15 g of fresh leaves with 150 mL sterile buffer (0.1 mol L^{-1} potassium phosphate buffer, containing 0.1% glycerol, 0.15% Tween 80, pH 7.0) (Xiong *et al.* 2021). The buffer with microbial cells was then passed through a 0.22 µm filter membrane and the DNA extraction was then performed using the DNeasy® PowerSoil® Pro-Kit (QIAGEN) according to the instructions. The primers used for bacterial 16S (V4–V5) gene amplifications were 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′). The PCR procedures and sequencing were described in Supplementary Note S2.

Statistical analysis

Tukey's Honestly Significant Difference (HSD) tests after one-way ANOVAs were used to compare individual differences among means at the significance level of $P < 0.05$. Leaf photosynthetic characteristics (P_n, g_s and E), physiological parameters (SOD and POD activity, MDA and total chlorophyll content) and alpha diversity of phyllosphere bacterial community were subjected to two-way ANOVAs to evaluate the impact of sex, pesticide and their interactions.

Alpha diversity including Simpson and Shannon indices was calculated by vegan and picante packages in R (Kembel *et al.* 2010; Oksanen *et al.* 2019). Significance analysis of differences among groups in phyllosphere bacterial community structure on the OTU abundances based on Bray–Curtis distances, including Adonis, Anosim and MRPP analysis were implemented with the phyloseq package in R (McMurdie and Holmes 2013). Bray–Curtis dissimilarities were calculated and principal coordinate analysis (PCoA) were used to visualize phyllosphere bacterial community differences between sexes and pesticide effects. A PerMANOVA model was also implemented to discern the variation attributed to sex, pesticide application and their interactions.

Two-way ANOVAs were used to analyse how sex, pesticide application and their interactions influence taxon abundances in phyllosphere bacterial community at phylum and genus levels. The picante and NST packages were conducted for analysis of the assemblage process of the phyllosphere bacterial community (Kembel *et al.* 2010; Ning *et al.* 2019). The prediction of phyllosphere bacterial community function was performed with FAPROTAX database (1.1) and Python (2.7), and we used the Scheirer–Ray–Hare test with rcompanion package in R to determine whether the sex or pesticide application influence the scale of functional prediction (Mangiafico and Mangiafico 2017). Finally, the principal component analysis (PCA) for leaf photosynthetic characteristics, physiological parameters and the relative abundances of two on phylum level was conducted by psych, ggplot2 and ggbiplot R packages (Revelle 2017; Vu 2016; Wickham 2016).

RESULTS

Effects of λ-cyhalothrin on photosynthesis characteristics and leaf physiological traits of *P. cathayana* females and males

Both sex and pesticide application affected the photosynthetic characteristics. Therein, net photosynthetic rate, stomatal conductance and transpiration rate were significantly higher in males than females. Pesticide application had a net negative effect on the abovementioned leaf physiological parameters. Only the net photosynthetic rate was affected by the interaction of pesticide and sex, with males being more negatively affected by pesticide application than females (P_n decreased by 19.26% in male plants and 15.51% in female plants) (Table 1).

Plant sex affected leaf SOD and POD activities as well as the total chlorophyll concentration. The application of λ-cyhalothrin significantly affected all leaf physiological parameters: leaf SOD and POD activity, leaf MDA and total chlorophyll concentration (Fig. 1). The response of leaf SOD and POD activities, leaf MDA and total chlorophyll concentration to pesticides were significantly different between females and males, where pesticide application significantly increased activity of leaf SOD and POD, and decreased total chlorophyll concentration, with no changes in leaf MDA concentration in females (Fig. 1). However, males increased leaf POD activity and MDA concentration, decreased the total chlorophyll concentration and showed no difference in leaf SOD activity (Fig. 1). These results indicated various regulations in response to the pesticide between sexes.

Effects of λ-cyhalothrin on composition of phyllosphere bacterial community

The phyllosphere bacterial community's alpha diversity (Simpson and Shannon) was unaffected by pesticide and sex (Supplementary Fig. S1). While the PCoA indicated that the first and second principal components explained 32.6% of the variation of four groups, and the bacterial composition of phyllosphere differed between females and males, as demonstrated by the PerMANOVA test ($R^2 = 0.21$, $P < 0.01$, Fig. 2a). The variation partitioning analysis showed that 9.92% variation of bacterial composition was explained by the plant sex, while the application of pesticide, and sex × pesticide interactions had no significant effects (Fig. 2b). Furthermore, three complementary non-parametric multivariate statistical tests (Adonis, Anosim and MRPP) indicated that the phyllosphere bacterial community composition was impacted by plant sex and the interactions of sex and pesticide ($P < 0.01$, $P < 0.001$, Table 2), while the microbe community were not significantly altered by the application of λ-cyhalothrin ($P > 0.05$, Table 2).

The taxonomic composition of the bacterial communities at the phylum and genus level (relative abundances > 0.01%) are shown in Fig. 3a and b. The bacterial reads were mostly assigned to 14 phyla and 13 genera based on the classifiable sequences. At the phylum level, the relative abundances of Acidobacteria and Gemmatimonadetes were significantly influenced by plant sex, and both were higher in males than in females (Fig. 3c). While the relative abundance of Actinobacteria and Proteobacteria was strongly affected by pesticide application, which were increased and decreased with the application of λ-cyhalothrin, respectively (Fig. 3c). At the genus level, the relative abundances of *Acinetobacter*, *Limnobacter* and *Acidovorax* were significantly affected by plant sex with males higher than females (Fig. 3d). Whereas the relative abundances of *Acinetobacter*, *Bacillus* and *Acidovorax* were greatly influenced by the interaction of sex and pesticide application (Fig. 3d).

Overall, heterogeneous selection explained 30.80% of the phyllosphere bacterial community assembly (Fig. 4a). Plant sex had significant effect on community construction than pesticide application. Heterogeneous selection accounted for a greater proportion of males. Bacterial community construction was more susceptible to pesticide application in males relative to

Table 1: Net photosynthetic rate (P_n), stomatal conductance (g_s) and transpiration rate (E) in *Populus cathayana* females and males as affected by application of λ-cyhalothrin (λ-CY) and control (CK)

Sex (S)	Pesticide (P)	P_n (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})
Female	CK	9.80 ± 0.31 c	0.14 ± 0.015 c	3.18 ± 0.29 bc
	λ-CY	8.28 ± 0.24 d	0.12 ± 0.019 c	2.49 ± 0.34 c
Male	CK	15.16 ± 0.50 a	0.36 ± 0.024 a	6.39 ± 0.87 a
	λ-CY	12.24 ± 0.15 b	0.27 ± 0.018 b	4.82 ± 0.45 ab
P values	Sex	***	***	***
	Pesticide	***	*	*
	S × P	*	ns	ns

Each value is means ± SE ($n = 6$). According to ANOVA, columns with the same letter are not significantly different at $P < 0.05$ followed by Tukey HSD tests. *, *** and ns indicate effects in the two-way ANOVA at $P < 0.05$, $P < 0.001$ and $P > 0.05$, respectively.

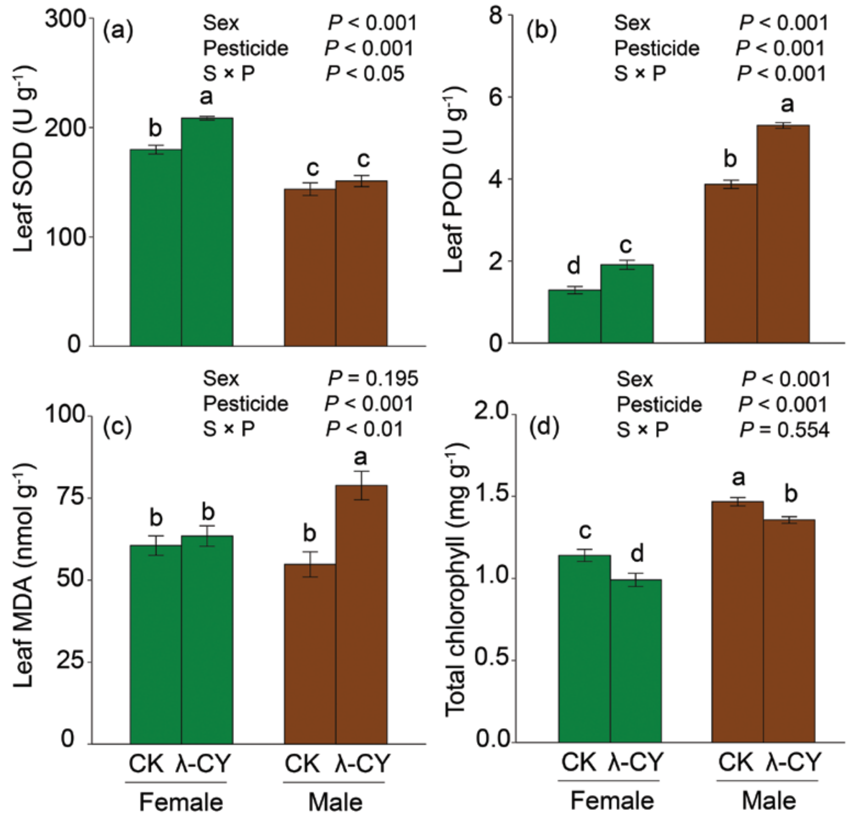

Figure 1: Effects of pesticide application on the activity of leaf SOD (**a**), leaf POD (**b**), content of leaf MDA (**c**) and total chlorophyll (**d**) of *Populus cathayana* females and males. Error bars, ± SE (n = 6). According to ANOVA, columns with the same letter are not significantly different at $P < 0.05$, followed by Tukey HSD tests. Abbreviations: CK = control treatments, λ-CY = λ-cyhalothrin applications.

females. Among others, pesticide application reduced the selection effect (Fig. 4b).

FAPROTAX was adopted to annotate the ecological functions of the phyllosphere bacterial communities (Supplementary Fig. S2). Plant sex mainly influenced the function of aromatic compound degradation and intracellular parasites, with the relative abundances in males being higher than in females (Supplementary Fig. S2). While the application of pesticide only affected the function of predatory or exoparasitic. Interactions of sex and pesticide strongly affected eight functions of bacterial communities: nitrate denitrification, nitrite denitrification, nitrous oxide denitrification, denitrification, dark hydrogen oxidation, nitrite respiration, nitrate respiration and nitrogen respiration, where the relative abundance of functions in females were all higher under λ-cyhalothrin application (Supplementary Fig. S2).

Relationships between leaf physiological traits and phyllosphere bacterial community

PCA revealed variation in the leaf photosynthetic characteristics, leaf physiology and phyllosphere bacterial communities of *P. cathayana* females and males in response to the application of λ-cyhalothrin (Fig. 5). The first and second PCA axes explained 57.2% and 21.6% of the total variation, respectively (Fig. 5). The relative abundance of Proteobacteria was positively correlated with leaf photosynthetic characteristics and negatively associated with leaf SOD activity and MDA content. The relative abundance of Actinobacteria was contrary to Proteobacteria (Fig. 5). The leaf physiological traits and phyllosphere bacterial community of females and males showed significant differences as indicated by the PerMANOVA test (R^2 = 0.794, $P < 0.001$, Fig. 5).

DISCUSSION

Our study revealed the impact of pesticide application on phyllosphere microorganisms of dioecious *P. cathayana* for the first time. We found that the alpha diversity of the phyllosphere bacterial community in *P. cathayana* was not affected by pesticide application or sex identity of the plant. In contrast, the phyllosphere bacterial community showed sex-specific responses.

Effect of plant sex and pesticide application on leaf physiology of *P. cathayana*

Genetic variation, environmental and anthropogenic factors such as herbicides, soil and phyllospheric microbial communities, and availability of resources can significantly affect plants' physiological activity (Sehrawat *et al.* 2022). Photosynthesis is the fundamental energy-conversion process on earth and plays a decisive role in plant growth. In our study,

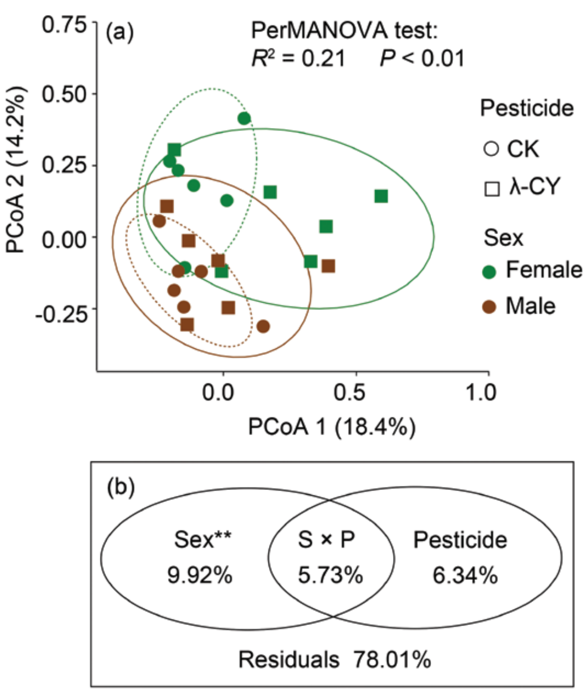

Figure 2: PCoA of phyllosphere bacterial community in *Populus cathayana* females and males under pesticide treatments (**a**). Values on PCoA axes indicate the percentages of total variation explained by each axis. Variation under application of pesticides of phyllosphere microbes explained by sex and pesticide was characterized by R^2 in the PerMANOVA (**b**). ** indicates significant effects at $P < 0.01$. Abbreviations of pesticide treatment can refer to Fig. 1.

Table 2: Changes in the composition of phyllosphere bacterial community of *Populus cathayana* females and males affected by λ-cyhalothrin (λ-CY) applications and control treatments (CK) by Adonis, Anosim and MRPP tests based on the Bray–Curtis distances

	Adonis		Anosim		MRPP	
Factors	R^2	P	R	P	δ	P
Sex (S)	**0.087**	**<0.01**	**0.206**	**<0.01**	**0.83**	**<0.01**
Pesticide (P)	0.062	0.051	0.078	0.089	0.842	0.056
S × P	**0.205**	**<0.01**	**0.245**	**<0.01**	**0.811**	**<0.001**

$P < 0.05$ in bold.

photosynthesis was consistently greater in males than in females and was not affected by pesticide application. Different resource-related tradeoffs between females and males can be attributed to the differential resource allocation where females allocate more resources to reproductive organs than males (Xia *et al.* 2020b, 2022; Zhang *et al.* 2019). Females have a higher photosynthetic rate than their male counterparts under favourable conditions, whereas males have a higher photosynthetic rate than females under relatively unfavourable environment conditions (Zhang *et al.* 2014). The soil we used in our experiment was comparatively nutrient-poor, and therefore, may have led to higher photosynthetic rate for males. On the other hand, our results were consistent with previous findings that pesticide application could significantly negatively influence the leaf photosynthetic apparatus (Lei *et al.* 2020; Qian *et al.* 2013; Wen *et al.* 2017). Chlorophyll is a primary photosynthetic pigment and is closely related to photosynthesis (Khalid *et al.* 2017). The total chlorophyll content of female and male plants decreased after pesticide application, which explained the reduction in photosynthesis. Meanwhile, pesticide application reduced stomatal conductance, which is corroborated by previous reports that Dichlorprop (DCPP) resulted in the tighter arrangement of epidermal cells and higher stomatal closure in *Arabidopsis* leaves (Qiu *et al.* 2022).

Our results showed that the MDA content of leaves after pesticide application was significantly increased in males but not in females. Meanwhile, both SOD and POD activities increased in leaves after pesticide application. The lipid peroxidation on the cell membrane caused damage to cells of *P. cathayana* leaves under pesticide stress, which resulted in the activation of antioxidant enzymes such as SOD and POD by ROS (Lei *et al.* 2020). Previous studies also showed that *P. cathayana* males exhibited stronger physiological adjustment than females under abiotic disturbances such as nutrient deficiency and heavy metal stress (Chen *et al.* 2013; Zhang *et al.* 2014).

Sex-specific phyllosphere bacterial community composition

Phyllosphere microbes are influenced by both intrinsic factors of host plant and environmental conditions (Xia *et al.* 2021; Zhu *et al.* 2022). In the present study, we found that some bacterial groups in the phyllosphere were more conservative and were unaffected by both sex of plant and pesticide application. For instance, the relative

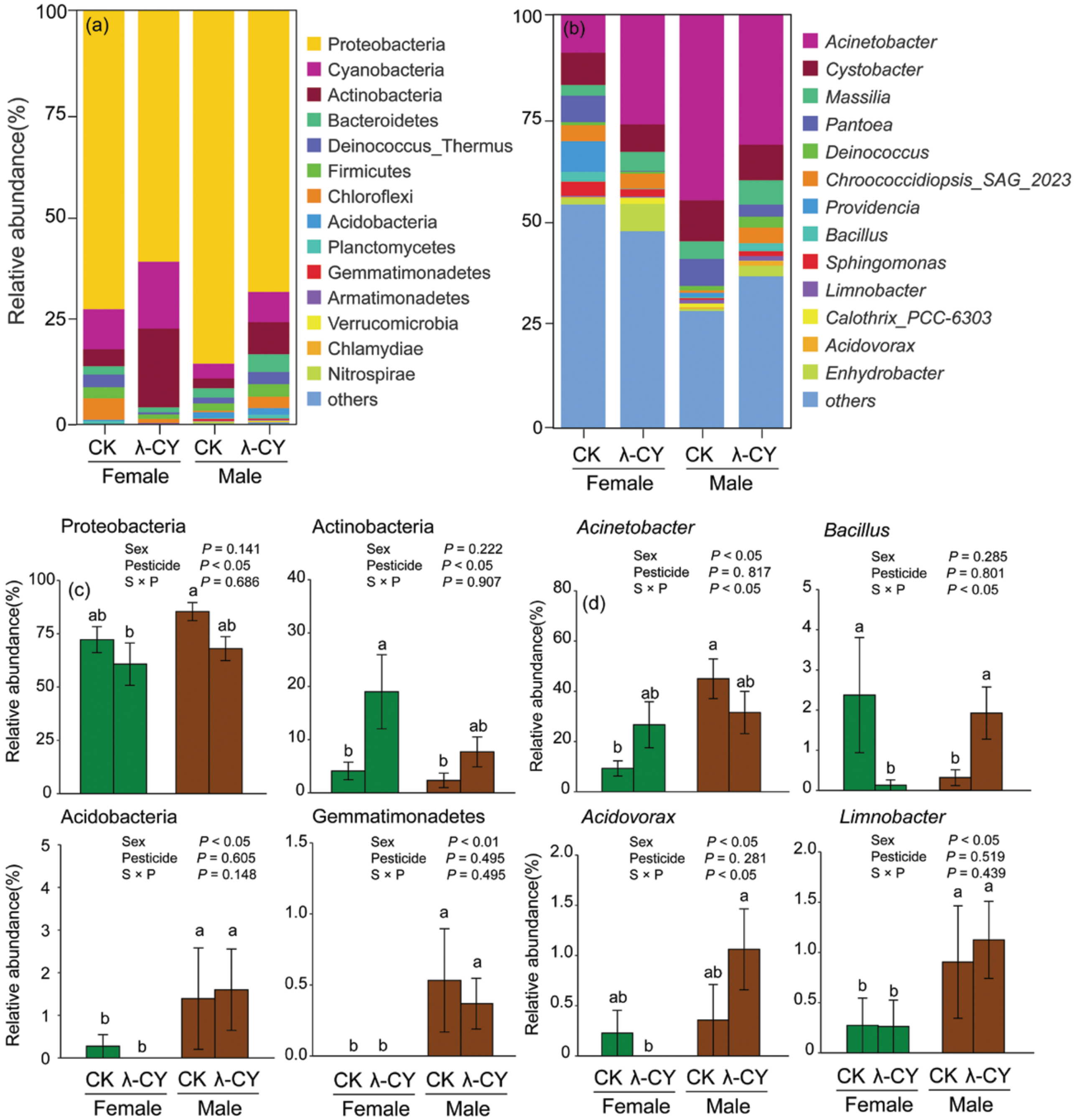

Figure 3: The relative abundances of dominant bacterial phyla (**a**) and genera (**b**). Two-way ANOVA models test the relative abundances of phyla (**c**) and genera (**d**) significantly affected by sex, pesticide application or their interactions. Columns with the same letter are not significantly different at $P < 0.05$ according to ANOVA, followed by Tukey HSD tests. Abbreviations of pesticide application can refer to Fig. 1.

abundance of Proteobacteria and Actinobacteria at the phylum level dominated the phyllosphere microbes of *P. cathayana*. These two bacteria were particularly suitable for persistence on the leaf surface as high abundance colonizers. Our results showed no significant difference in the alpha diversity of phyllosphere bacterial communities between females and males which was in line with a recent study (Liu *et al.* 2021a). Simultaneously, the variability in interspecific microbial community composition among species is much greater than the intraspecific variability in the same habitat (Redford *et al.* 2010). The phyllosphere of plants is a habitat of high microbial diversity, but also it is a dynamic microenvironment due to its direct exposure to various abiotic and biotic factors (Kinkel 1997).

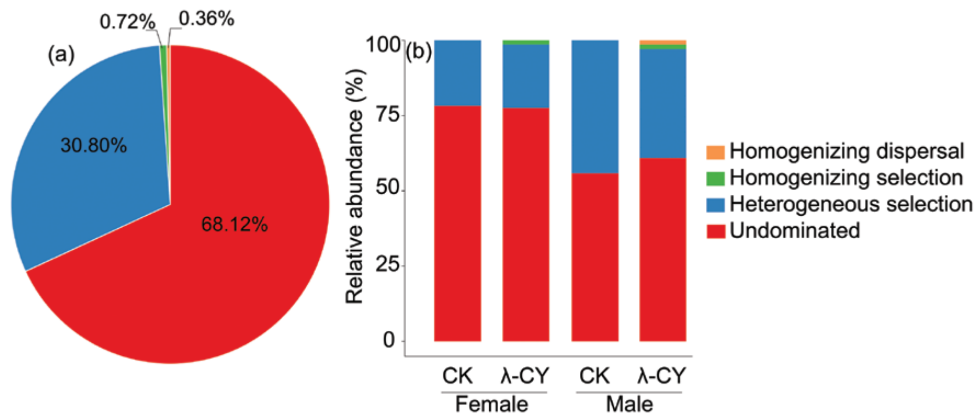

Figure 4: Relative dominance of phyllosphere bacterial community assembly processes. Overall dominance of assembly processes (**a**) and relative dominance of assembly processes within pesticide application between sexes (**b**). Abbreviations of pesticide treatment can refer to Fig. 1.

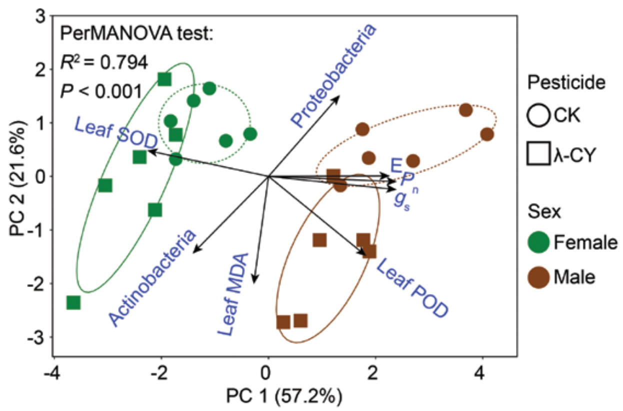

Figure 5: PCA of leaf physiological traits and phyllosphere bacterial community of *Populus cathayana* females and males under pesticide application. Leaf physiological traits and phyllosphere bacterial community corresponding to the arrows are shown as follows: the SOD activity (leaf SOD), the POD activity (leaf POD), the MDA content (leaf MDA), net photosynthetic rate (P_n), stomatal conductance (g_s), transpiration rate (E), the relative abundance of the Proteobacteria and the Actinobacteria phylum.

However, the application of pesticide did not affect the alpha diversity of phyllosphere bacteria. The results associated with the assessment of bacterial community diversity indicated a relatively high resilience to the applied pesticide. The resistance of foliar microbial communities to pesticides has been previously confirmed in relevant studies (Perazzolli *et al.* 2014; Yashiro and McManus 2012). However, the beta diversity of the phyllosphere bacterial community demonstrated that the variation of microbial community composition between sexes was larger than that of pesticide application, suggesting a more important role of sex selection on plant phyllosphere microbial community.

Environmental and genetic factors together determine microbial community assembly in leaves and soils (Dincă *et al.* 2022; Yang *et al.* 2022). Diffusion and selection are the two main forces in the primary ecological processes of community assembly, with drift and diversification also being instrumental. Dove *et al.* (2021) reported that the assembly of the *Populus* microbiome is determined by time, genotype and habitat, which was regulated by both selection and stochastic factors. In our study, males had fewer diffusion constraints and stronger environmental selection than females.

Proteobacteria are often the most dominant bacterial communities in the phyllosphere at the phylum level (Bashir *et al.* 2022) and are generally associated with nutrient availability (Liu *et al.* 2021a). The relative abundance of Proteobacteria in the phyllosphere was higher for male plants than female plants. In contrast, the relative abundance of Actinobacteria was higher for females than in males. The phyla of Actinobacteria exhibit diverse physiological and metabolic characteristics, such as the production of extracellular enzymes and the formation of varies secondary metabolites (Alvarez *et al.* 2017). These characteristics may result in the photosynthesis of females encountering fewer adverse effects after consecutive pesticide application than males. In addition, pesticide application significantly affected phyllosphere bacterial taxa at the genus level (Cernava *et al.* 2019; Perazzolli *et al.* 2014). Some specific bacterial genera such as *Acinetobacter* and *Bacillus* have been reported to have quorum quenching ability in the phyllosphere of tobacco plants (Ma *et al.* 2013). Bacterial quorum quenching was associated with bacterial biofilm formation and pathogenesis of certain phytopathogenic bacteria.

Acinetobacter belongs to the phylum Proteobacteria, while *Bacillus* has a strong growth-promoting effect on rice (*Oryza sativa*) with potential antibacterial activity and high antagonistic activity (Bashir *et al.* 2022). These correlate with the functions projected by FAPROTAX in our results, such as denitrification and respiration.

Correlations analysis between leaf physiological traits and phyllosphere bacterial community

The PCA of leaf physiological traits and phyllosphere microbial community composition showed significant sex-specific differences. Photosynthetic efficiency was the main factor responsible for the differences in phyllosphere microbial community between males and females, while activities of SOD and POD differentiated females and males, respectively. Phyllosphere microbiome was closely associated with leaf functional traits. Photosynthesis and antioxidant chemicals indirectly and directly mediated microbial growth and development (Friesen *et al.* 2011). The results of our common garden experiment demonstrated that plant leaf physiological traits and sex were crucial mediation factors of phyllosphere bacterial community composition and assembly. In the future, it is necessary to study the effects of various pesticides on the phyllosphere microbial community in dioecious plants. Also, more leaf functional traits are needed to consider.

CONCLUSIONS

This study reveals that the leaf physiology and phyllosphere microbe of dioecious *P. cathayana* respond distinctly to the application of pesticide. Photosynthetic apparatus and leaf physiological traits of *P. cathayana* were significantly affected by sex, pesticide application and their interactions and these effects were more pronounced in males than females. Moreover, plant sex had a greater effect on phyllosphere bacterial community composition and community assembly than pesticide application. However, pesticide application affected the relative abundance of specific bacterial taxa where Proteobacteria was negatively affected and Actinobacteria was positively affected at the phylum level. These shifts in phyllosphere bacterial community composition appeared to have a unique ecological function, which could be reflected in the physiological adjustment of leaves. Our results provide further evidence that leaf physiology and plant sex are among the key determinants of the phyllosphere bacterial community composition of *P. cathayana*, which should improve our understanding of the sex discrimination of microorganisms in the dioecious plants.

Supplementary Material

Supplementary material is available at *Journal of Plant Ecology* online.

Figure S1: The Simpson (a) and Shannon (b) diversities of phyllosphere bacterial community in *P. cathayana* females and males under pesticide application.

Figure S2: The functional prediction of phyllosphere bacterial community of *P. cathayana* females and males under pesticide application.

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Authors' Contributions

Zuodong Zhu: investigation. Yue He: data curation, formal analysis, investigation, methodology, software, visualization, writing—original draft. Jiahui Xu: investigation. Zhenghu Zhou: investigation. Amit Kumar: writing, reviewing and editing. Zhichao Xia: conceptualization, funding acquisition, resources, project administration, supervision, validation, writing—review and editing.

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

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