

Original Research

Sexual Differences in Growth and Physiological Characteristics of *Populus cathayana* under Pb Stress and *Leucoagaricus* sp. Colonization

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Abstract

Populus cathayana was used as a model species to investigate sexual differences in plant growth and physiological responses to lead (Pb) stress (0, 150 and 300 mg Pb²⁺ kg⁻¹ dry soil) with mycorrhizal (Myc) fungi colonization by *Leucoagaricus* sp. Results showed that high concentration of Pb stress (300 mg/kg) caused disorder in photosynthesis, inhibited plant biomass, changed antioxidant enzyme activities and increased Malondialdehyde (MDA) content in both sexes, particularly in females. Male saplings showed greater biomass, gas exchange capacity, antioxidant enzyme activity and lower MDA content when exposed to Pb stress alone and to a combination of Pb and Myc fungi treatment. Furthermore, *Leucoagaricus* sp. inoculation alleviated Pb toxicity in both sexes by increasing biomass accumulation, promoting gas exchange capacity, enhancing antioxidant enzyme activities and decreasing relative electrolyte leakage under low concentration (150 mg/kg) and high concentration Pb stress. Inoculation with *Leucoagaricus* sp. promoted Pb uptake in both sexes, and induced the sequestration of more toxic Pb in the root systems in males. Therefore, *P. cathayana* males associated with *Leucoagaricus* sp. performed best under high concentration of Pb stress and more suitably restore Pb-polluted soils than inoculated females due to the higher Pb uptake capacity and greater growth traits.

Keywords: mycorrhizal fungi, heavy metal, phytoremediation, dioecy, *Populus cathayana*

Introduction

Heavy metal contamination has become one of the most important environmental problems globally due to its irreversible and high toxicity to living organisms [1]. Lead (Pb) is classified as a group of nonessential heavy

metals that are harmful to plants even at very low concentrations [2, 3]. Pb stress cause a series of adverse effects on plants; for example, Pb toxicity can result in changes in antioxidant enzyme activities, increases in membrane permeability and the disturbance of mineral nutrition balance, ultimately resulting in lower growth and biomass accumulation in plants [4, 5]. Meanwhile, excess Pb causes adverse effects on photosynthesis activity, including decreases in photosynthetic pigments

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and the distortion of chloroplast ultrastructure [6-8]. Meanwhile, Pb can accumulate in plants, although it is not necessary for plants. Therefore, phytoremediation using plants that remove metals from the environment or reduce their toxicity has made rapid progress in the clean-up of metal-polluted areas in a cost-effective and environmentally friendly manner [9, 10]. Additionally, plant growth in adverse environments can interact with various microbial communities, such as mycorrhizal (Myc) fungi and bacteria [11, 12]. All these microorganism communities might play an important role in plant adaptation to the heavy metal stress. Plants associated with mycorrhizal fungi-mediated phytoremediation are highly sought after. Previous studies have reported mycorrhizal fungi can protect tree roots against toxic metals in *Pinus tabulaeformis* [13] and *Populus canescens* [3]. The genus *Leucoagaricus* is widely distributed in China and North America, which belong to the family Agaricaceae [14]. Among *Leucoagaricus naucinus* (Fr.) Singer is a mycorrhizal fungus also known as *Leucoagaricus leucothites* and abundant in the conifer forests [15, 16]. Previous studies suggest that *L. leucothites* have highest free radical scavenging ability and anti-oxidant activity and growing on the metal-rich soils [17, 18]. However, limited knowledge is available on the interactions between *Leucoagaricus* sp. and plants to improve the capacity of plants to adapt to metal-contaminated soils.

Dioecious plants are an important component of terrestrial ecosystems, representing 6.0% of angiosperm species and constituting 4% of vascular plants [19, 20]. Some studies have reported that dioecious plants have sex-specific adaptations to heavy metal contamination [6, 21], and males and females usually show distinct morphological [22, 23], physiological [22, 24], and ecological traits [25] as result of different reproduction demands. For example, male plants of *Morus alba* showed greater chlorophyll pigment concentrations, antioxidant enzyme activity and lower MDA content and relative electrolyte leakage than females when exposed to Pb stress [26], and male *Silene latifolia* individuals indicated higher growth and reproduction than females growing in soil polluted with either Cu or Cd [27].

Populus cathayana is a typical deciduous tree that is widely distributed in northern, central and southwestern China and has been used as a suitable candidate for the phytoremediation of heavy metal-polluted soils due to its tolerance and accumulation of high concentrations of heavy metals [28-30]. Several studies have indicated sexual differences in the morphology, physiology, biochemistry, and ultrastructure of *Populus* under heavy metal stress [6, 31, 32]. However, little is known about whether there are sex-specific responses to Pb stress and mycorrhizal fungi colonization. Hence, *P. cathayana* was used to investigate sex-specific differences in growth and physiological progress in

response to Pb stress, *Leucoagaricus* sp. colonization and their combination. Based on the existing knowledge of sex-specific responses to adverse conditions, we further hypothesized that i) males have greater tolerance under Pb than females; ii) *Leucoagaricus* sp. fungi colonization can reduce Pb toxicity in both sexes but to a different degree; and iii) *Leucoagaricus* sp. fungi colonization can improve the phytoremediation efficiency of *P. cathayana* males.

Materials and Methods

Biological Materials and Experimental Design

P. cathayana cuttings were collected from the healthy annual shoots of female and male trees located in their natural habitats in Sining, Qinghai Province, China. All cuttings were sterilized with 4% NaClO solution for 30 min and then rinsed three times by sterile deionized water. The cuttings were cultured in fine sand (autoclaved at 121°C for 2 h), and after sprouting and taking root for about four weeks, 96 male and 96 female healthy cuttings of *P. cathayana* with similar heights and root lengths (the longest root was approximately 10 cm) were selected for the present study.

The culture substrate consisted of autoclaved (121 °C for 2 h) soil and fine sand in a 1:1 (V/V) ratio. The soil was collected from the nursery garden of Northwest A&F University and screened through a 2 mm sieve. The sands were also screened and then washed three times with running water. After being mixed thoroughly, 6 kg of culture substrate was added into a 5 L plastic pot.

The mycorrhizal fungus *Leucoagaricus* sp. was isolated from mushrooms distributed under a poplar on the campus of Northwest A&F University in Yangling, Shaanxi Province, China. The fungus was cultured in liquid potato dextrose broth (PDB) for 14 d (26°C, 120 rpm) in the dark. Then, the mycelium was collected from the liquid medium and rinsed three times with sterile deionized water. Finally, 100 g of collected wet mycelium was suspended again into 1000 ml of sterile deionized water and blended to prepare a slurry (30 s under the highest speed). The slurry was used as the fungus inoculum in this study. When transplanting the cuttings into each pot, 20 ml of inoculum was inoculated onto the surface of roots for inoculated treatments, whereas for non-inoculated treatments, the surface of roots was inoculated with the same amount of sterilized mycelial suspension.

Forty-five days later, colonization of *Leucoagaricus* sp. into the *P. cathayana* roots was checked and confirmed, and half of the cuttings were exposed to Pb stress. Deionized water containing 48 mM Pb(NO₃)₂ was evenly added to surface of the culture substrate until the Pb²⁺ concentrations reached 0, 150 and 300 mg per kg⁻¹ of dried culture substrate. In total, there

were twelve subgroups in this study, combined with two types of sex, two fungal inoculation conditions (inoculated or non-inoculated), and three degrees of Pb stress (0, 150 and 300 mg Pb²⁺ kg⁻¹ dry soil), and each subgroup contained 8 individual plants. All of the plants were placed outdoors in a randomized complete block design but protected from rain with plastic film. During the experimental period, the mean monthly temperature and relative humidity were 24-26°C and 47-80%, respectively.

After being treated with Pb for two months, the plants were harvested, and then the following parameters were measured. For each parameter, at least five individuals from each subgroup were used for the determinations.

Estimation of the Mycorrhizal Colonization Rate

The fresh roots of *P. cathayana* plants were cleaned carefully with deionized water. More than 200 root segments (1 cm, diameter < 1 mm) were collected randomly from each tested plant. The segments were bleached with 10% KOH at 90°C for 30 min, acidified in 1% HCl for 10 min and then stained with 0.05% trypan blue in lactophenol at 90°C for 20 min. Finally, the mycorrhizal colonization rate was estimated according to the grid line intersect method [33].

Plant Growth Measurements

After being treated with Pb for two months, five cuttings were randomly chosen from each treatment for the determination of biomass. All samples were carefully washed thoroughly in tap water, and the plants were separated into leaves, stems, and roots. The samples were soaked in 0.2% EDTA for 30 min to eliminate possible chemical contamination and were then rinsed with deionized water. The dry biomass of leaves (LM), stems (SM) and roots (RM) was measured after samples were oven-dried at 70°C for 48 h. The total dry biomass was calculated based on the dry biomass of LM, SM and RM.

Gas Exchange Parameters and Chlorophyll Content Measurements

Net photosynthesis (P_n), stomatal conductance (g_s), intercellular CO₂ concentration (C_i) and transpiration rate (E) of tested plants were measured by using the third and fourth fully expanded leaves with a Li-6400 portable photosynthesis system (Li-Cor, Lincoln NE, USA) from 8:00 to 11:30 am. The flow rate through the sample chamber was set at 1000 mL.S⁻¹, the leaf temperature was 25±0.5°C, and the ambient CO₂ concentration was 400±5 mmol.mol⁻¹.

Fresh leaf samples were cleaned with deionized water to remove any surface contamination, and

100 mg of fresh sample was homogenized in 25 ml of acetone (80%) in the dark at room temperature for 10 h. A UV/Vis spectrophotometer (UV-1800, Shimadzu, Japan) was used to measure the content of photosynthetic pigments at 646, 663 and 470 nm. Chlorophyll contents were calculated from equations derived by [34]. The total chlorophyll content (T_{chl}) was the sum of chlorophyll a (*Chl a*) and chlorophyll b (*Chl b*).

Determination of Pb Concentration

Oven-dried plant samples were ground into a fine powder and passed through a 100-mesh screen. A representative sample of 0.5 g was digested with HNO₃-HClO₄ (5:1, V/V) in Teflon bombs in a microwave oven and subsequently diluted in deionized water to a final volume of 10 ml. The concentration of Pb in plant samples was measured by atomic absorption spectrophotometry (AA7000, Shimadzu, Japan). The Pb transfer index (*TI*) was calculated as the ratio of the Pb concentrations in the aboveground (stems and leaves) and belowground (roots) plant parts.

Malondialdehyde (MDA) Contents and Relative Electrolyte Leakage Determinations

The malondialdehyde (MDA) concentration was determined by the thiobarbituric acid method [35]. Fresh leaf samples (0.5 g) were ground to a fine powder in liquid nitrogen, homogenized in 10 ml of 10% trichloroacetic acid and centrifuged at 12,000 g at 4°C for 15 min. The supernatant was collected and mixed with 2 ml of 0.6% thiobarbituric acid, heated in a water bath at 100°C for 15 min, and cooled in an ice bath. The mixture was centrifuged for 10 min at 12,000 g. The absorbance of the supernatant was measured at 450, 532 and 600 nm.

Relative electrolyte leakage (REL) was measured as described by [36]. Fifteen fresh leaf discs (0.5 cm in diameter) were incubated in tubes with 10 ml of deionized water at 25°C for 10 h. The electrical conductivity of the bathing solution (*C1*) was determined using a conductivity instrument (LC116, Mettler-Toledo Instruments Co., Ltd, Shanghai, China). Then, the tubes were incubated in a boiling water bath (100°C) for 25 min and cooled to room temperature, and the total electrical conductivity (*C2*) was measured. Ion leakage was calculated using the following equation: REL = (*C1/C2*) × 100.

Enzyme Extractions and Activity Assays

Enzymes were extracted using potassium phosphate buffer. Briefly, fresh leaf samples (0.5 g) were ground to a fine powder in liquid nitrogen and then homogenized in 6 ml of 50 mM potassium phosphate buffer (pH = 7.6), which contained 0.1 mM ethylenediaminetetraacetic

acid (EDTA), 1% (W/V) polyvinylpyrrolidone (PVP), 0.1 mm phenylmethylsulfonyl fluoride (PMSF) and 0.2% (V/V) Triton X100. The homogenate was centrifuged at 12,000 g at 4°C for 15 min, and the supernatant was used for enzyme activity assays; all operations were performed at 0-4°C.

Superoxide dismutase (SOD) activity was determined by measuring the inhibition of the photochemical reduction in nitroblue tetrazolium (NBT) by the method of [37]. Catalase (CAT) activity was determined by the decomposition of H₂O₂ and was measured spectrophotometrically by assessing the decrease in absorbance at 240 nm [38]. Peroxidase (POD) activity was expressed as the amount of enzyme required to change the optical density by 0.001 per minute at 470 nm [38].

Determination of Proline Content

The proline assay was conducted according to [39]. Fresh leaf samples (0.5 g) were ground to a fine powder in liquid nitrogen and then homogenized in 10 ml of 3%

sulfosalicylic acid solution and centrifuged at 12,000 g at 4°C for 15 min. The supernatant was collected and reacted with 2 mg of ninhydrin reagent and 2 ml of pure acetic acid. The mixture was heated in a water bath at 100°C for one hour and cooled in an ice bath. Next, 4 ml of toluene was added to the mixture and shaken well for 20 s. The upper layer was collected, and the absorbance was measured at 520 nm.

Statistical Analyses

All results are presented as the means and standard deviations for the five repeats. Three-way ANOVAs were performed to evaluate the overall effects of sex, Pb, mycorrhizal fungi and their interactive effects. One-way ANOVAs was used to determine differences among treatments, and Tukey's multiple range test was employed to detect individual differences among means. Differences were considered significant at the P<0.05 level. In all cases, statistical analyses were conducted using SPSS 17.0 software.

Table 1. The mycorrhizal rate, leaf dry mass (LM), stem dry mass (SM), root dry mass (RM), total dry mass (TM) and shoot height (SH) of female and male *Populus cathayana* saplings in response to treatments of lead (Pb), *Leucoagaricus* sp., and their combination.

Sex	Pb stress (mg/kg)	Mycorrhizal fungi	Colonization Rate (%)	RM (g)	SM (g)	LM (g)	TM (g)	SH (cm)
Female	0	Non-Myc	-	0.55±0.03 cd	2.9±0.33	5.14±1.47 bc	8.6±1.74 bc	54.1±5.4 bcde
	0	Myc	57.79±5.04 c	1.06±0.09 a	3.18±0.7	5.09±0.59 bc	9.3±0.22 abc	65±7.2 abc
	150	Non-Myc	-	0.37±0.1 de	2.67±0.31	4.7±0.7 c	7.74±1.0 c	45±4.3 cde
	150	Myc	65.97±8.14 c	0.32±0.02 e	2.92±0.77	8.69±2.59 a	11.93±2.8 ab	45.8±2.2 cde
	300	Non-Myc	-	0.29±0.09 e	2.23±0.55	4.54±0.95 c	7.06±1.29 c	35±1.4 e
	300	Myc	41.63±8.15 d	0.38±0.03 de	2.24±0.4	5.26±0.8 bc	7.89±0.52 c	38±1.7 de
Male	0	Non-Myc	-	0.63±0.08 bc	2.46±0.42	4.68±0.09 c	7.77±1.36 c	55.8±4.6 bcde
	0	Myc	65.78±4.51 c	1.11±0.08 a	3.28±0.47	8.32±2.24 ab	12.7±2.44 a	80±4.33 a
	150	Non-Myc	-	0.74±0.06 bc	2.1±0.18	5.06±1.15 bc	7.86±0.99 c	65.8±6.5 abc
	150	Myc	77.68±5.78 b	0.74±0.03 bc	2.49±0.25	6.17±0.16 abc	9.4±0.33 abc	68.3±2.2 ab
	300	Non-Myc	-	0.33±0.03 e	2.26±0.76	4.04±0.33 c	6.62±0.3 c	40.8±3.2 de
	300	Myc	89.68±2.18 a	0.8±0.17 b	3.05±0.4	8.73±1.06 a	12.6±1.38a	57.5±4.3 bcd
		Pb stress (A)	2.36NS	77.65***	4.45NS	0.49NS	1.692NS	23.82**
		Myc fungi (B)	1416.27***	91.68***	8.262**	21.34***	27.608***	14.82***
		Sex (C)	50.32***	74.12***	0.39 NS	1.91 NS	2.302NS	32.22***
		A×B	4.25**	32.27***	0.16NS	0.77NS	1.661NS	3.34NS
		A×C	13.98***	13.12***	3.36 NS	3.84*	1.582NS	3.61 NS
		B×C	52.32***	6.35**	2.9 NS	0.99 NS	4.349*	2.46 NS
		A×B×C	18.2***	5.43**	0.32 NS	12.04***	13.161***	0.63 NS

Different letters indicates significant differences between treatment (mean±SE, n = 5) at P<0.05 according to Tukey's multiple range tests. Significance values of the factorial analysis (ANOVA) for the effects are denoted as follows: ns, non-significant; * P<0.05; **P<0.01; ***P<0.001.

Results

Sex-Related Difference in Mycorrhizal Colonization Rate and Plant Growth Characters

Under the of absence of Pb stress (0 mg/kg), high mycorrhizal colonization rate was observed in males (65.6%) and females (57.5%). Increasing Pb concentration to 150 mg/kg significantly increased mycorrhizal colonization in males (77.68%) and females (65.34%). However, high concentration of Pb stress (300 mg/kg) significantly decreased mycorrhizal colonization in females (41.67%), but not males (Table 1).

In response to Pb stress, RM, SM, LM and TM of male and female plants were reduced under 300 mg/kg Pb stress (Table 1); however, under 150 mg/kg Pb stress, RM, SM, LM and TM were increased in

males but decreased in females. Under the absence of Pb stress (0 mg/kg), the inoculation of significantly increased the RM and TM in male and female plants; however, the increase in TM in males (63.4%) was greater than that in females (8.1%). Moreover, under 300 mg/kg Pb stress, the colonization of *Leucoagaricus* sp. increased the RM, LM and TM by 9%, 15.9% and 11.8%, respectively, in females and by 142.4%, 116.1% and 90.3%, respectively, in males. Males had significantly higher values in these indices than females exposed to the combination of Pb and *Leucoagaricus* sp. treatment (Table 1).

Sex-Related Difference in Gas Exchange and Chlorophyll Pigments

Both male and female plants showed a lower P_n , g_s and E but higher C_i in response to Pb treatment

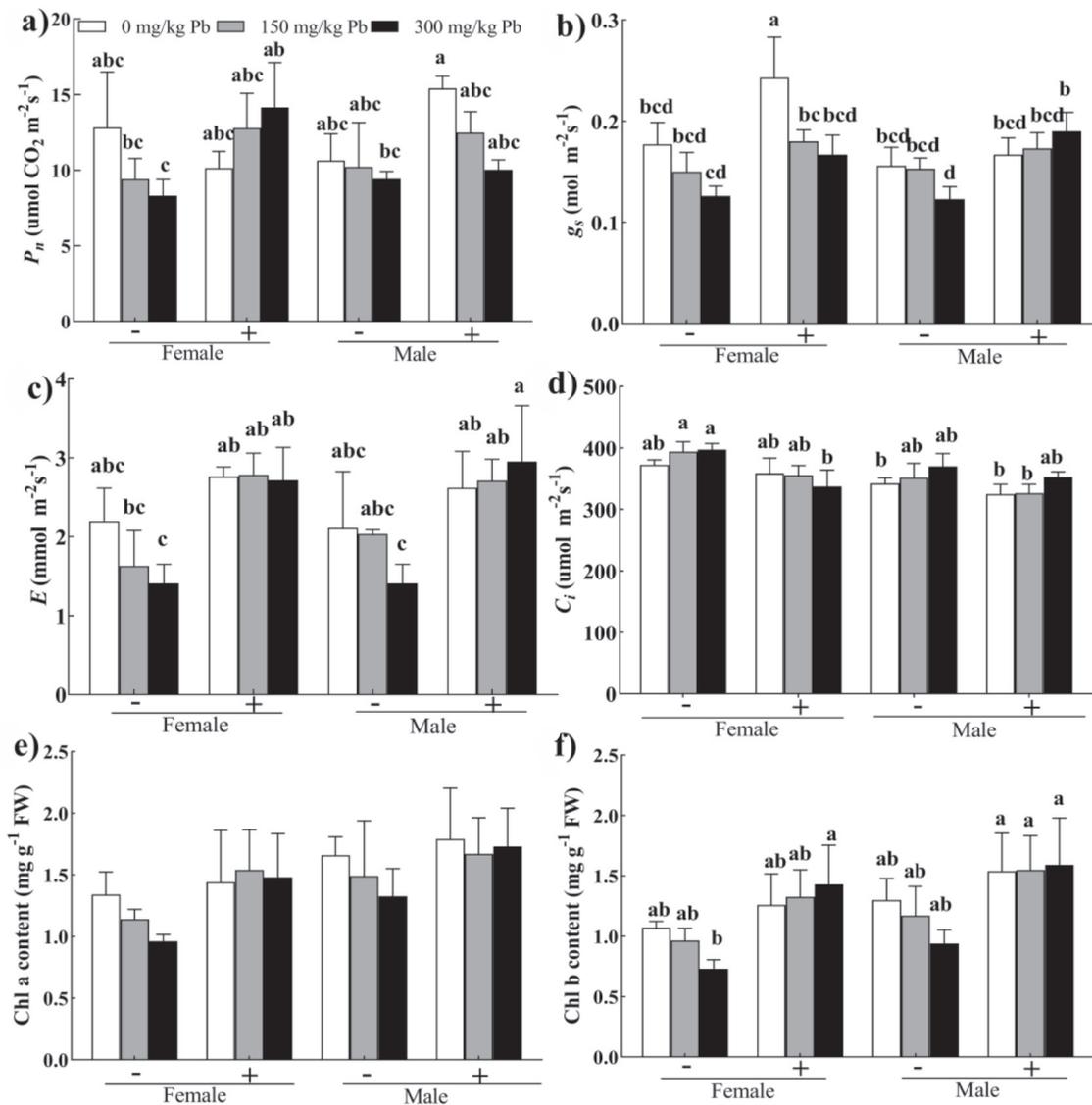


Fig. 1. Gas exchange and chlorophyll pigments of female and male *Populus cathayana* saplings in response to treatments of lead (Pb), *Leucoagaricus* sp. (-, without *Leucoagaricus* sp. inoculation; +, with *Leucoagaricus* sp. inoculation), and their combination. Each value is the mean \pm SE ($n = 5$). Different letters in the same column represent significant differences ($P < 0.05$).

compared with the control. Male plants showed a lesser decrease in P_n , g_s and E (5.7%, 33.17% and 21.5%, respectively) than females (35.3%, 35.9% and 28.8%, respectively) under 300 mg/kg Pb stress compared with the control. Under the absence of Pb stress (0 mg/kg), the inoculation of *Leucoagaricus* sp. significantly increased the P_n , g_s and E in males, but decreased the P_n in females. Moreover, under 150 mg/kg Pb stress, the colonization of *Leucoagaricus* sp. increased the P_n , g_s and E by 39.8%, 71.8% and 20% respectively, in females and by 21.4%, 33.5% and 12.5%, respectively, in males. However, the colonization of *Leucoagaricus* sp. decreased the C_i by 9.8% and 7.1%, respectively, in females and males under 150 mg/kg Pb stress (Fig. 1).

Compared to the control, low and high concentration Pb stress (150 and 300 mg/kg) significantly decreased *Chl a* and *Chl b* content in both male and female plants, but males had higher values of *Chl a* and *Chl b* content than females under Pb stress. Under unpolluted soil (0 mg/kg), the colonization of *Leucoagaricus* sp. significantly increased the *Chl b* content by 17.8% and 18.5% respectively, in female and male plants, and the increase in *Chl a* and *Chl b* content in males was greater than that in females (Fig. 1e,f). Under 300 mg/kg Pb

stress, *Leucoagaricus* sp. colonization significantly increased *Chl a* and *Chl b* content by 31.1% and 61.2% respectively, in male and 54.2% and 95.9% respectively, in female plants.

Sex-Related Differences in Pb Concentration

Compared with the control, in response to Pb treatment, male and female plants showed significant increases in the Pb concentration of leaves, stems, and roots, and males exhibited a higher Pb concentration in roots than females under low and high concentration of Pb stress (150 and 300 mg/kg Pb), whereas there was no significant difference between the two sexes under unpolluted treatment (Fig. 2a). Under the absence of Pb stress (0 mg/kg Pb), *Leucoagaricus* sp. inoculation significantly increased the Pb concentration in roots of male and female plants, but there was a slight decrease in the Pb concentration of leaves and stems in male and female plants. Under low and high concentration of Pb stress, *Leucoagaricus* sp. inoculation significantly increased the Pb concentration in roots but decreased the Pb content in leaves and stems in both sexes, and the increase in Pb concentration in roots of males (32.9% for 150 mg/kg Pb, 40.6% for 300 mg/kg Pb) was greater than that in females (17.2% for 150 mg/kg Pb, 17.6% for 300 mg/kg Pb) in combined Pb and *Leucoagaricus* sp. treatment. *TI* was significantly decreased in either sex with Pb treatment alone or with the interaction *Leucoagaricus* sp. treatment (Fig. 2b).

Sex-Related Differences in MDA and REL Determinations

Compared with the control, in response to Pb treatment, male and female plants showed a significant increase in MDA content and REL. Under 300 mg/kg Pb stress, *Leucoagaricus* sp. inoculation significantly increased the MDA content by 48.5% and 53.2% respectively, in male and female plants, compared with the control. However, females exhibited a higher MDA content than males under low and high concentration of Pb stress. Under the absence of Pb stress (0 mg/kg Pb), *Leucoagaricus* sp. inoculation significantly decreased the MDA content of male and female plants (Fig. 3a), whereas the REL of male and female plants was not significantly different under *Leucoagaricus* sp. (Fig. 3b). Under Pb treatment, *Leucoagaricus* sp. colonization significantly decreased MDA content and REL in male and female plants, and the decrease in MDA in females (20.2% for 150 mg/kg Pb, 23.1% for 300 mg/kg Pb) was greater than that in males (10.5% for 150 mg/kg Pb, 11.7% for 300 mg/kg Pb).

Sex-Related Differences in Antioxidant Defense Systems

Compared with the control, in response to 150 mg/kg Pb treatment, male and female plants showed

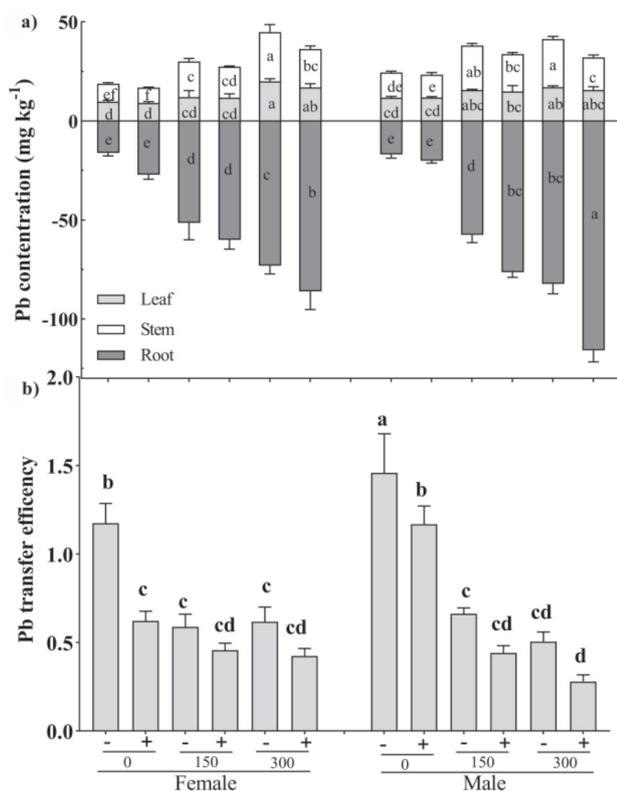


Fig. 2. The lead (Pb) contents a) and Pb b) transfer of female and male individuals of *Populus cathayana* in response to treatments of Pb, *Leucoagaricus* sp. (-, without *Leucoagaricus* sp. inoculation; +, with *Leucoagaricus* sp. inoculation), and their combination. Each value is the mean \pm SE ($n = 5$). Different letters in the same column represent significant differences ($P < 0.05$).

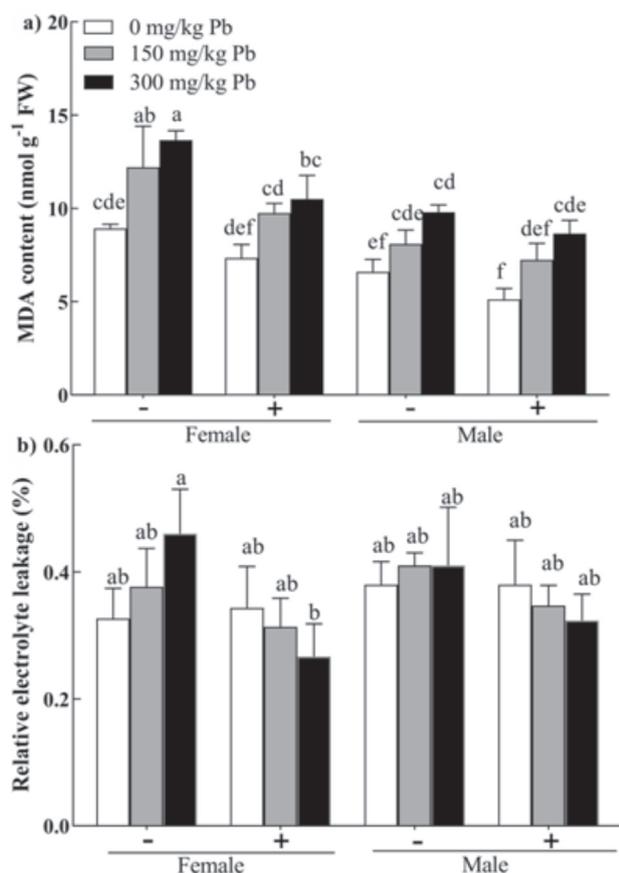


Fig. 3. a) Malondialdehyde (MDA) and b) relative electrolyte leakage (REL) of female and male individuals of *Populus cathayana* in response to treatments of lead (Pb), *Leucoagaricus* sp. (-, without *Leucoagaricus* sp. inoculation; +, with *Leucoagaricus* sp. inoculation), and their combination. Each value is the mean±SE ($n = 5$). Different letters in the same column represent significant differences ($P < 0.05$).

significant increases in SOD, CAT, and POD activity, whereas sexually divergent responses to 300 mg/kg Pb treatment were found in SOD, CAT and POD activity; male seedlings showed a significant increase in SOD (26.9%) and CAT (140%) activity, but the activities of SOD (11.7%) and CAT (30.6%) activity were decreased in female seedlings in response to 300 mg/kg Pb stress (Fig. 4a, b). Under unpolluted soil (0 mg/kg), *Leucoagaricus* sp. inoculation significantly increased the activities of SOD, CAT and POD activity in male and female plants. Under low and high concentration of Pb treatment (150 and 300 mg/kg), *Leucoagaricus* sp. colonization significantly increased SOD and CAT activity in both sexes under 150 and 300 mg/kg Pb stress and increased POD activity only in males under 300 mg/kg Pb addition, while males showed significantly higher SOD, CAT and POD activity under Pb treatment alone or combination with *Leucoagaricus* sp. treatment (Fig. 4c).

In response to Pb treatment, male and female plants showed a significant increase in proline content, and the increase in proline in females (95.6% for

150 mg/kg Pb, 102.5% for 300 mg/kg Pb) was greater than that in males (6.4% for 150 mg/kg Pb, 46.4% for 300 mg/kg Pb). under the absence of Pb stress (0 mg/kg), *Leucoagaricus* sp. inoculation significantly increased the proline content by 50.3% and 24.5% respectively, in male and female plants, and males showed a higher proline content than females under *Leucoagaricus* sp. treatment (Fig. 4d). Under low and high concentration of Pb treatment (150 and 300 mg/kg Pb), *Leucoagaricus* sp. colonization induced a significant increase in proline content in males (20.4% for 150 mg/kg Pb, 14.3% for 300 mg/kg Pb) and females (25.9% for 150 mg/kg Pb, 17.2% for 300 mg/kg Pb).

Discussion

Previous studies have reported that sexually dimorphic plants may have sex-specific adaptations to heavy metal stress, with most of these studies indicating that males exhibited a greater adaptive capacity than females when exposed to heavy metal stress [6, 21, 26]. Our results indicated sex differences in biomass accumulation, photosynthetic capacity, Pb concentration, and antioxidant defense systems in *P. cathayana* males and females under Pb stress. When compared to females, males were better adapted, which was visible as higher root biomass, P_n , leaf *Chl a* and *b*, antioxidant enzyme activity and proline contents and as lower MDA and REL under Pb stress. In our study, Pb stress (300 mg/kg) decreased the biomass and growth in male and female plants; however, at a low concentration (150 mg/kg), Pb stress only inhibited growth and biomass in females, not in males, and the inhibition of growth and biomass accumulation can be considered an index of plant tolerance (Table 1). Plant growth is closely linked with intrinsic physiological processes, including photosynthesis and metabolism [7]. In our study, a low net photosynthetic rate and chlorophyll pigment content were observed in both male and female plants under Pb treatment, but under the same stress, P_n decreased more in females than in males (Fig. 1), which is in agreement with previous observations in *P. cathayana*, *Thespesia populnea* and *Raphanus sativus* [6, 40, 41].

Damage to the plasma membrane is the most obvious phenomenon induced by oxidative stress in plants exposed to heavy metal stress [42, 43]. REL reflects the extent of cellular membrane damage as well as MDA, which is an important product of membrane lipid peroxidation [32, 44]. Previous studies have suggested that females exhibited higher REL and MDA content than males under heavy metal stress [27, 31]. In agreement with these results, *P. cathayana* males showed lower REL and MDA content than females under Pb stress in this study (Fig. 3a, b). This suggests that *P. cathayana* males may have a better mechanism to protect the cellular membrane than females under heavy metal stress. The increase in oxidative stress also induces the expression of antioxidant systems by

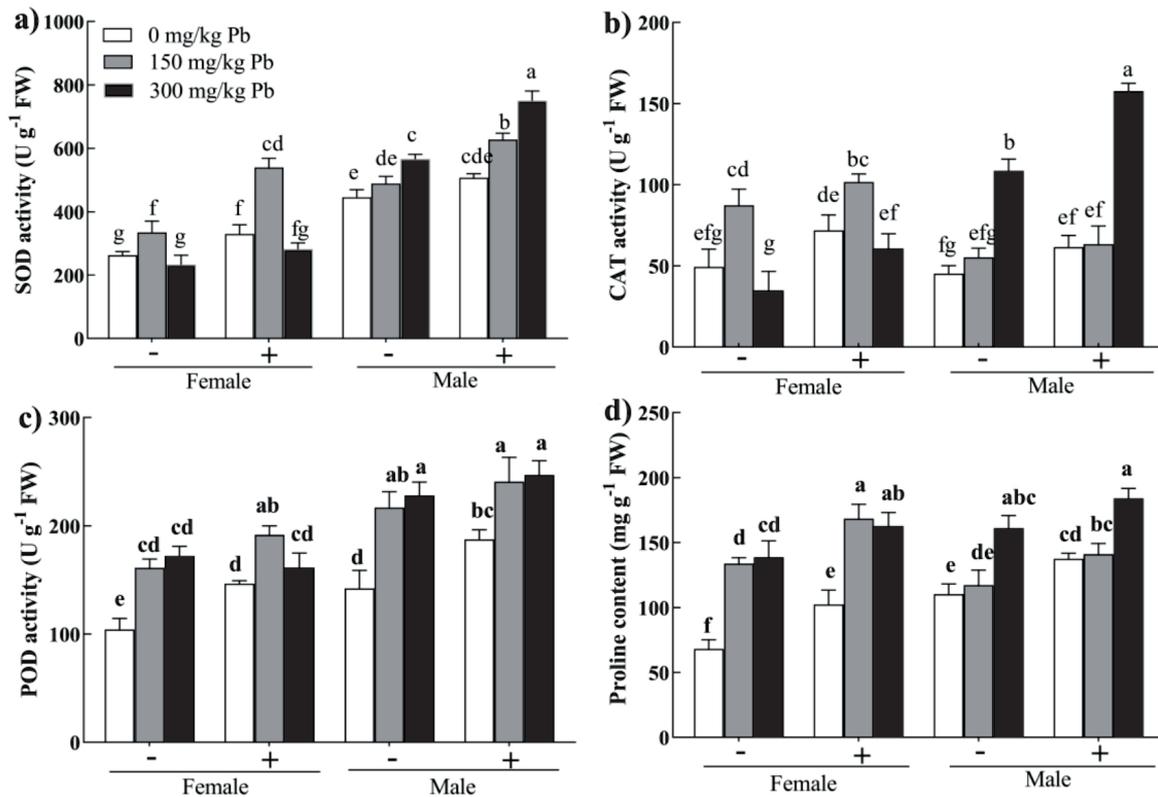


Fig. 4. a) Activities of superoxide dismutase (SOD), b) catalase (CAT), c) peroxidase (POD) and d) proline content of female and male individuals of *Populus cathayana* in response to treatments of lead (Pb), *Leucoagaricus* sp. (-, without *Leucoagaricus* sp. inoculation; +, with *Leucoagaricus* sp. inoculation), and their combination. Each value is the mean \pm SE ($n = 5$). Different letters in the same column represent significant differences ($P < 0.05$).

signalling molecules [21]. SOD, CAT and POD activity significantly increased under a low concentration of Pb (150 mg/kg) in male and female plants; however, SOD and CAT activity decreased under a high concentration of Pb (300 mg/kg) in female plants but not in males (Fig. 3a,b). Moreover, males always showed higher SOD and POD activity than females, suggesting that male plants have a higher free radical scavenging capacity than female plants. Indeed, proline is a major constituent of osmoregulation in the leaves of many plant species, and increases in proline content are beneficial for a plant cell to adapt to adverse environments [45]. Males showed higher proline content than did females under Pb stress (Fig. 4d), and indeed, Tripathi and Gaur [46] also confirmed that proline has hydroxyl radical scavenging capacity. Therefore, *P. cathayana* males were the lower sensitive to the variations of Pb concentration than females because they possess a more efficient antioxidant system and higher photosynthesis capacity to alleviate Pb stress, particularly in the higher concentration of Pb stress.

Our results indicated that the exotic *Leucoagaricus* sp. successfully colonized roots of both sexes of *P. cathayana*, the mycorrhizal colonization rate was increased in response to Pb stress in *P. cathayana* males confirms the tolerance of heavy metal to this fungus, and thus, the *Leucoagaricus* sp. might have a potential

application in the phytoremediation of polluted soils by heavy metal. In this study, sex-specific in mycorrhizal colonization rate response to Pb stress might be related to the sensitivity both of the fungi species and of the host to metal stress, and on the interaction between the fungal species and the host [47]. Furthermore, our results suggested that *Leucoagaricus* sp. colonization significantly increased biomass accumulation, *Chl a* and *b* content, SOD and CAT activity, proline content, and Pb concentration in roots and decreased MDA content and Pb concentration in the aerial parts (stems and leaves), implying that *Leucoagaricus* sp. fungi colonization can mitigate Pb-induced toxicity. Previous studies have also shown that mycorrhizal symbiosis contributes to alleviating metal cation toxicity for the host plant by improving plant growth and reducing lipid peroxidation [3, 13, 48]. Indeed, *Leucoagaricus* sp. colonization significantly increased the Pb concentration in roots and decreased Pb transfer from roots to shoots, particularly in males. Increased heavy metal concentrations in mycorrhizal fungi-colonized plants have been found in previous studies [13, 49], which can mainly be ascribed to the chelation of Pb by fungal exudates and modified Pb ion mobility in the root apoplast [50, 51], and the prevention of Pb transfer to the shoots, thereby mitigating the toxic effect on sensitive leaves by Pb stress.

Conclusions

Our study revealed that *P. cathayana* had significant sexual differences in biomass accumulation, enzyme activity, MDA content and Pb accumulation in response to Pb stress and *Leucoagaricus* sp. colonization. Males are more tolerant of Pb stress than females, owing to better biomass accumulation and antioxidant enzyme activities and lower ion electrolyte leakage when exposed to Pb alone. Meanwhile, *Leucoagaricus* sp. fungi colonization alleviated the phytotoxic effects of Pb by promoting gas exchange capacity, antioxidant enzyme production and biomass accumulation and restricting Pb transfer to the shoots in both sexes, and the alleviation effects were greater in females than males. Moreover, compared to non-mycorrhizal males, mycorrhizal males exhibit a higher biomass accumulation and more Pb uptake in root, without obvious negative effects on growth and physiological traits. Therefore, males in association with *Leucoagaricus* sp. can be considered as an appropriate candidate for the remediation of soils polluted by Pb, due to their greater tolerance and phytoremediation ability.

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Conflict of Interest

The authors declare no conflict of interest.

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