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# Nitrogen addition alleviated sexual differences in responses to cadmium toxicity by regulating the antioxidant system and root characteristics, and inhibiting Cd translocation in mulberry seedlings

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## ABSTRACT

Cadmium (Cd) toxicity and nitrogen (N) deposition are two major environmental stresses which can affect plant growth. It's less clear that how the combined Cd accumulation and N deposition affect the male and female plants of dioecious species. The aim of the present study was to detect sex-specific responses to Cd stress and simulated N deposition in one-year-old male, female and hermaphrodite seedlings of *Morus alba*. Changes in morphology, physiology, root architecture and biomass of the three sex types of mulberry seedlings were determined. The results showed that Cd toxicity caused limited growth, impaired photosynthetic apparatus and decreased gas exchange rates with significant sex-specific differences. Mulberry was found to deploy detoxification mechanisms to avoid or tolerate toxic Cd effects through the activation of the antioxidant system, increasing proline and non-protein thiol contents, translocating Cd into different plant parts and decreasing biomass. Females displayed a low tolerance to high Cd and were more sensitive to Cd stress. Simulated N deposition alleviated the negative effects of Cd on leaves and decreased sex-specific differences in the three kinds of mulberry seedlings, but N fertilizer did not affect the total biomass. The N-stimulated increasing in proline and non-protein thiol contents might play a crucial role in resisting the damage caused by Cd stress, and the three kinds of mulberry seedlings had slightly different ways of improving Cd tolerance by N deposition. Sexual differences in Cd accumulation are correlated with root architecture. This study provides evidence for the utilization of mulberry to treat Cd-contaminated soils under N deposition.

## 1. Introduction

Cadmium (Cd) is one of the most toxic heavy metal elements in soil and regarded as a serious ecological problem due to its high toxicity in humans (Rizwan et al., 2017). During recent years, Cd contents in arable soils have dramatically increased due to human activities, such as rapid industrialization, wide application of fertilizers and wastewater irrigation of agricultural lands (El Rasafi et al., 2020). Cadmium can accumulate within plant organs and cause several physiological changes when taken up by roots (Gill et al., 2012; Wang et al., 2019b). Cadmium limits plant growth and development by decreasing plant biomass and disrupting the normal metabolism of cells and the activity of various protective enzymes (He et al., 2017). Atmospheric nitrogen (N)

deposition is another serious environmental problem. N deposition has substantially increased owing to the rapid expansion of industry and intensive agricultural activities during the last few decades (Reay et al., 2008). N deposition can improve photosynthesis and biomass in plants under N deficiency conditions (Gaju et al., 2016). However, excessive N deposition may disturb nutrient distribution in plants and soils (Peng et al., 2019), and cause a negative impact on ecosystems (Roth et al., 2020; Shen et al., 2020).

Some studies have investigated the combined effect of Cd and N on plant growth (Wångstrand et al., 2007; Song et al., 2019; Wang et al., 2020; Yang et al., 2020). Nitrogen could increase the Cd exchange capacity and bio-available Cd content in soils, and enhance Cd uptake and accumulation in plants (Yang et al., 2020). On the other hand, N also

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improves the tolerance of plants to Cd by regulating cell wall isolation and oxidative resistance, and producing more glutathione (GSH), phytochelatin (PCs) and nitric oxide (NO) (Yang et al., 2020).

These previous studies on the combined effect of Cd and N deposition have focused on monoecious plants, while males and females of dioecious plants usually respond differently to environmental pollution (Melnikova et al., 2017; Chen et al., 2018). Females usually have a higher resource demand because of a greater reproductive investment compared to males. This reproductive pattern is conducive to meeting the resource demands of different sexes and effectively reducing intra-specific sex competition (Munné-Bosch, 2015). However, the long-term adaptive evolution of dioecious plants with different reproductive costs in different environments leads to differences in stress sensitivity. Many studies have found that females display higher sensitivity than males under stress conditions. Such sex-specific responses lead to contrasting changes in the morphology, physiology, growth, survival, and reproductive patterns of male and female plants. For example, the males of *Corema album*, *Urtica dioica*, *Populus cathayana* and *Populus yunnanensis* grow better than females under drought or heavy metal stress conditions (Chen et al., 2011, 2013; Simancas et al., 2016; Díaz-Barradas et al., 2018). Most previous studies have investigated sex-specific responses to a single stress, but only few have focused on the combined effects of two or more stresses on plant growth, even though this is more likely to occur in nature. However, some studies have shown that males have a lower tolerance to unfavorable conditions than females (Juvany and Munné-Bosch, 2015; Ruuhola et al., 2018).

Mulberry (*Morus alba* L.), an important traditional industrial crop, has been cultivated widely and for a long time in China. With a rapid growth rate, strong adaptability to adverse environments and high tolerance to metals, mulberry has recently attracted more attention (Yang et al., 2017; Lei et al., 2019; Zeng et al., 2019). As a dioecious species, mulberry shows sex-specific responses to a single stress, such as UV-B radiation, and female trees are affected more negatively than males (Zhang et al., 2020a). Interestingly, there are both dioecious and hermaphrodite mulberry species in the nature, and there are differences in the responses of *M. alba* males, females and hermaphrodites to environmental stresses. The objective of the present study was to highlight sex-specific responses to Cd toxicity and tolerance in the three sex forms of mulberry seedlings (male, female and hermaphrodite) under N deposition. We compared changes in the morphology, physiology, root architecture and biomass of the three sex forms of mulberry saplings under N and Cd stress. Our aim was to answer the following questions: (1) whether Cd causes sex-specific adaptive responses on mulberry growth, and (2) whether N alleviates damage induced by Cd stress in sex-specific adaptive responses? This work will provide background knowledge for developing management options with the objective of treating Cd-contaminated soils under N deposition using mulberry plantations.

## 2. Materials and methods

### 2.1. Plant materials and experimental design

One-year-old healthy cuttings were collected from mature trees representing *Morus alba* L. cv. Nongsang 14 (male), *Morus alba* L. cv. Qiangsang 1 (female) and *M. alba* L. cv. Nongsang 12 (hermaphrodite) at the Sericultural Research Institute, Zhejiang Academy of Agricultural Sciences. Cuttings (about 15 cm in length) were planted in the field for rooting in March 2018. After sprouting and growing for 45 days, 80 healthy rooted cuttings of each sex with approximately equal heights (about 20 cm) were transplanted into plastic pots of 40 cm (height) × 26 cm (diameter). Each pot was filled with 9 kg of soil with or without cadmium. The properties of the soil used in this study were as follows: pH 5.18, total N 0.73 g·kg<sup>-1</sup>, organic carbon 12.4 g·kg<sup>-1</sup>, hydrolysable N 57.3 mg·kg<sup>-1</sup>, available phosphorus 20.1 mg·kg<sup>-1</sup>, and available potassium 71.0 mg·kg<sup>-1</sup>.

As reported in recent studies, the increasing atmospheric N deposition rate in eastern China has reached 83.3 kg N ha<sup>-1</sup>·yr<sup>-1</sup> with the intensified human activities (Xu et al., 2015; Ti et al., 2018). In addition, previous studies found that mulberry yield can reach the highest level at about 200 kg N ha<sup>-1</sup>·yr<sup>-1</sup> (Xu, 2013). Therefore, according to the projected value of local N deposition by the year 2050 (Galloway et al., 2004), two N levels, no N addition (N0 or control) and N addition of 90 kg N·ha<sup>-1</sup>·yr<sup>-1</sup> (N90), were applied as N treatments in this study. Correspondingly, in some mines of southern China, cadmium concentrations in the soil can be as high as 35.7–323.8 mg·kg<sup>-1</sup> (Huang and Zhao, 2017). Previous studies had showed that mulberry exhibits tolerance to soil Cd concentration less than 75.8 mg·kg<sup>-1</sup>, and the mulberry seedlings would gradually die when the soil Cd concentration reach 145 mg·kg<sup>-1</sup> (Jiang et al., 2019; Guo et al., 2021). Therefore, two Cd levels, no Cd addition (Cd0) and Cd addition of 100 mg·kg<sup>-1</sup> (Cd100), were applied as Cd treatments. A 2 × 2 × 3 factorial design experiment was conducted to study the effects of two N levels (N0 and N90), two Cd levels (Cd0 and Cd100), and three sexes (males, females and hermaphrodites) on gas exchange, chlorophyll fluorescence, antioxidants, proline, non-protein thiols, biomass, and root phenotype. In total, 12 different treatments were designed. In each treatment, 20 cuttings of each sex as replicates were used to minimize sampling errors. The treatments lasted 90 days from June to August.

At the beginning of the experiment, the Cd solution was prepared by dissolving appropriate quantities of CdCl<sub>2</sub>·2.5 H<sub>2</sub>O (Chemical Co. Ltd., China) in distilled water (pH 7.0), and then it was evenly added to the dry soil in the pot before transplanting seedlings. There was a plastic tray under each pot for collecting the soil leachate which was poured from the tray back into the pot to avoid Cd loss. Based on the atmospheric N deposition rate of 90 kg N ha<sup>-1</sup>·yr<sup>-1</sup>, we calculated the average weekly N deposition rate is 24.66 mg·m<sup>-2</sup>. According to each pot area (0.053 m<sup>2</sup>) in this experiment and N content of NH<sub>4</sub>NO<sub>3</sub> (0.35 mg·mg<sup>-1</sup>), the day spraying amount of NH<sub>4</sub>NO<sub>3</sub> for each pot was 3.73 mg. After transplanting into pot for 15 days, seedlings were irrigated with simulated N deposition for 90 days. NH<sub>4</sub>NO<sub>3</sub> was mixed with 20 L of deionized water and sprayed evenly onto seedlings using an electric sprayer twice a week over the entire experimental period, and seedlings with N0 treatment were sprayed with the same amount of distilled water.

The cuttings were grown in a natural greenhouse under ambient conditions with a daytime temperature of 19–28 °C, a night-time temperature of 12–18 °C, and a relative humidity of 40–85%. During the experiment, the places of pots were often randomly switched to avoid differences in microclimate. The experiments were conducted in the Botanical Garden of the Zhejiang A&F University (119° 44' E, 30° 16' N), East China. The area has a monsoonal subtropical climate with a mean annual precipitation of 1420 mm and a mean annual temperature of 15.6 °C, ranging from 24 °C in July to 3 °C in January.

### 2.2. Chlorophyll fluorescence and gas exchange measurements

After 90-day treatments, four seedlings of each sex in each treatment were randomly selected for relative chlorophyll content (SPAD), chlorophyll fluorescence, and gas exchange measurements from 8:00 a.m. to 11:30 a.m. SPAD was measured using a portable chlorophyll meter (SPAD-502 plus, KoNicaMiNolt, Japan). Five readings were taken between the base and apex of each leaf blade (avoiding the veins), and the mean SPAD reading values were recorded (Perveen et al., 2015). Chlorophyll fluorescence measurements were performed on the four fully opened leaves from the terminal leaflets using a portable chlorophyll fluorometer (PAM-2500 WALZ, Germany). The minimal fluorescence ( $F_0$ ) of photosystem II (PSII) was measured using an analytical light at 0.05 μmol m<sup>-2</sup>·s<sup>-1</sup> of photon flux density (PFD) after 40 min of dark-adaptation. Subsequently, a saturating pulse (8000 μmol m<sup>-2</sup>·s<sup>-1</sup> PFD, 2 s) was administered to measure the maximal fluorescence ( $F_m$ ). Then, active light (197 μmol m<sup>-2</sup>·s<sup>-1</sup> PFD) was turned on to enable the

leaves to undergo photosynthesis, and a saturated pulse (8000  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  PFD) was performed every 20 s to observe the changes in various fluorescence parameters. After 300 s, the steady-state value of the maximum photochemical efficiency of PSII ( $F_v/F_m$ ), the effective quantum yield of PSII ( $Y(II)$ ) and non-photochemical quenching ( $q_N$ ) were recorded by the instrument. Gas exchange parameters were measured for the same leaves using an LI-6800 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). The conditions were as follows: a leaf temperature of 25 °C, relative air humidity of 50%,  $\text{CO}_2$  concentration of  $400 \pm 5 \mu\text{mol}\cdot\text{mol}^{-1}$ , and light intensity of  $1000 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ . When steady-state gas exchange rates were achieved under these conditions, the net photosynthesis rate ( $A$ ), stomatal conductance ( $g$ ), transpiration ( $Tr$ ), and intercellular  $\text{CO}_2$  concentration ( $C_i$ ) were recorded.

### 2.3. Antioxidant measurements

After 90-day treatments, four seedlings of each sex in each treatment were selected and 0.5 g of fresh leaves from each seedling were collected for measuring the enzyme activities according to Liu et al. (2019). Leaves were ground in liquid nitrogen, followed by extracting with 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1% (w/v) PVP, 0.1 mM PMSF and 0.2% (v/v) Triton X100 for the measurement of SOD, POD and CAT. Then the homogenate was centrifuged at 14 000g at 4 °C for 15 min, the supernatants were collected for the enzyme activity assays.

The total superoxide dismutase (SOD) activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Habiba et al., 2015). One unit of SOD activity was determined as the quantity of enzyme that induced 50% prohibition of photochemical reduction of the NBT. Peroxidase (POD) activity was measured according to the method of Tauqeer (Tauqeer et al., 2016). One unit of POD was determined as the amount of enzyme producing 1  $\mu\text{mol}\cdot\text{min}^{-1}$  of oxidized guaiacol at 470 nm. Catalase (CAT) activity was determined by measuring the decomposition of  $\text{H}_2\text{O}_2$  at 240 nm ( $0.04 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ ) (Habiba et al., 2015).

The malondialdehyde (MDA) determination principle is that the degradation products of lipid peroxide can condense with thiobarbituric acid (TBA) to form the red product, which has the maximum absorption peak at 532 nm (Jia et al., 2017). Fresh leaves (0.5 g) with 10% trichloroacetic acid (TCA) was centrifuged at 12,000 rpm for 10 min. Then, 2 mL of 0.6% TBA in 10% TCA was added to a 2-mL aliquot of the supernatant. The mixture was heated in boiling water for 15 min and then quickly cooled in an ice bath. After centrifugation at 10000g for 10 min, the absorbance of the supernatant was determined at 450, 532 and 600 nm using a spectrometer. The following formula was used to calculate the concentration of MDA:  $C (\mu\text{mol}\cdot\text{L}^{-1}) = 6.45 \times (A_{532} - A_{600}) - 0.56A_{450}$ .

### 2.4. Proline and non-protein thiol measurements

Proline was measured according to the acid ninhydrin method (Silva and Matos, 2016). Four seedlings of each sex in each treatment were selected and 0.2 g of fresh leaves from each seedling were ground in liquid nitrogen and then extracted with 3% sulfosalicylic acid. An aliquot of each extract (2 mL) was incubated with 2 mL of ninhydrin reagent (2.5% ninhydrin, 60% glacial acetic acid, and 40% 6 M phosphoric acid) and 2 mL of glacial acetic acid at 100 °C for 30 min. The reaction was terminated in an ice bath. Then, 5 mL toluene was added and vortexed, followed by incubation at 25 °C for 24 h. The absorbance was measured at 520 nm using a spectrophotometer.

Non-protein thiols were measured, as described by Bhoomika et al. (2014). Fresh leaves (0.5 g per seedling) from four replicates of each treatment were homogenized in 6.67% 50-sulpho salicylic acid. After centrifugation at 10,000g for 10 min at 4 °C, the supernatant was reacted with Ellman's reagent. The absorbance was measured at 412 nm

using a spectrophotometer.

### 2.5. Root architecture characters, and biomass and Cd measurements

After 90-day treatments, four replicated seedlings were harvested from each sex in each treatment, washed thoroughly with deionized water, and separated into roots, leaves and stems. The root system of each seedling was removed, the water on the surface was wiped by tissue paper, and then it was scanned using an automated scanner (LA2400, Seiko Epson Corp, Japan). The total root length, root surface area, root volume, root tip number, and root diameter classes were evaluated using a WinRHIZO Root Analyzer System (Version 2012b, Regent Instruments Inc., Quebec, Canada). Then, all tissues were dried in an oven at 80 °C for 48 h to reach a constant dry weight and weighed, and the dry mass accumulation was calculated.

Parts of dried samples were ground to fine powder and passed through a 100-mesh screen for Cd content measurements by ICP-AES (PerkinElmer Inc., Shelton, CT, USA). All measurements were performed for four replicates per treatment. Cd translocation factor ( $\text{Cd}_{\text{TF}}$ ) in plants was calculated using the following formula (Zacchini et al., 2009):

$$\text{Cd}_{\text{TF}} = \frac{\text{Cd content in stems and leaves}(\text{mg}\cdot\text{kg}^{-1})}{\text{Cd content in roots}(\text{mg}\cdot\text{kg}^{-1})} \quad (1)$$

Cd bioconcentration factor ( $\text{Cd}_{\text{BCF}}$ ) in plants was calculated using the following formula:

$$\text{Cd}_{\text{BCF}} = \frac{\text{Cd content in plants (or in roots, stems, leaves)}(\text{mg}\cdot\text{kg}^{-1})}{\text{Cd content in the soil}(\text{mg}\cdot\text{kg}^{-1})} \quad (2)$$

### 2.6. Statistical analyses

Data were analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA), including one-way and three-way ANOVAs. Three-way ANOVAs were used to evaluate the effects of Cd, N deposition, sex, and their combinations. Within each sex, one-way ANOVAs were used to determine differences among treatments. For ANOVAs, the data were tested for normality and equality of variances and, if necessary, natural log transformations were performed. Significant differences were determined at the 95% significance level.

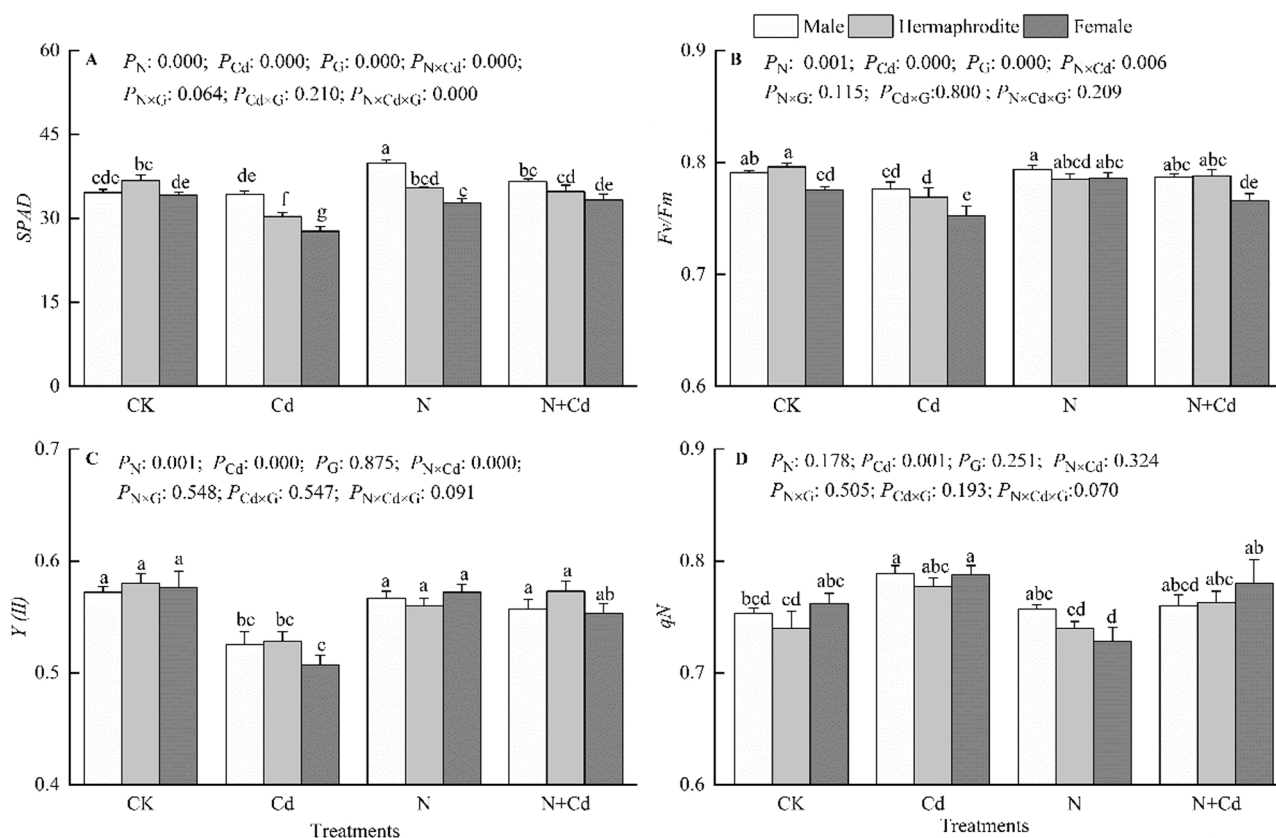
## 3. Results

### 3.1. SPAD and chlorophyll fluorescence parameters

There was a reduction in SPAD under Cd treatment in females and hermaphrodites (Fig. 1A). N addition increased the SPAD values of males, while the combined N + Cd treatment had no significant effect on SPAD compared to the control. Cd treatment significantly reduced  $F_v/F_m$  and  $Y(II)$  of the three kinds of mulberry seedlings, and the decrease in males was less than that in females and hermaphrodites ( $P < 0.05$ ). N addition had no significant effect on  $F_v/F_m$  and  $Y(II)$  of the three kinds of mulberry seedlings. On the other hand, the increase in  $q_N$  in males exposed to the Cd treatment was significant ( $P < 0.05$ ). However, there were no significant differences in  $F_v/F_m$ ,  $Y(II)$  and  $q_N$  of the three kinds of mulberry seedlings under the combined N + Cd treatment compared with those in control conditions. There were significant interaction effects between Cd and N on these chlorophyll fluorescence parameters, except for  $q_N$ .

### 3.2. Gas exchange parameters

Cd treatment decreased  $A$  significantly in the three kinds of mulberry seedlings compared to the control plants ( $P < 0.05$ ) (Fig. 2A), with a greater decrease in females (39.94%) and hermaphrodites (28.00%) compared to plants in control conditions. N treatment increased the net



**Fig. 1.** Effects of cadmium (Cd), nitrogen addition (N) and their combination on the relative chlorophyll content (SPAD) (A), maximum photochemical efficiency of PSII ( $F_v/F_m$ ) (B), effective quantum yield of PSII ( $Y(II)$ ) (C), and nonphotochemical quenching ( $q_N$ ) (D) in *Morus alba* males, hermaphrodites and females. CK, control plants. Different letters represent statistically significant differences between treatments (mean  $\pm$  SE,  $n = 4$ ) at  $P < 0.05$  according to least significant difference (LSD) multiple range tests.  $P_N$ , N effect;  $P_{Cd}$ , Cd effect;  $P_G$ , sex effect;  $P_{N \times Cd}$ , the interactive effect of N and Cd;  $P_{N \times G}$ , the interactive effect of N and sex;  $P_{Cd \times G}$ , the interactive effect of Cd and sex;  $P_{N \times Cd \times G}$ , the interactive effect of N, Cd and sex.

photosynthesis rate (A) significantly in males and hermaphrodites compared to the control plants ( $P < 0.05$ ). The decrease in  $g$  (Fig. 2B) and  $Tr$  (Fig. 2D) in males was also less than that in females and hermaphrodites under Cd treatment (Fig. 2D). On the other hand, when these seedlings were exposed to the combined treatment (N + Cd), sex-specific differences decreased compared with those in plants exposed to Cd alone. There was no significant interaction effect between Cd and N on the gas exchange parameters.

### 3.3. Malondialdehyde and antioxidant enzymes

The malondialdehyde contents and antioxidant enzymes activities in different treatments were showed in Fig. 3. There was a significant effect on the MDA content in the three kinds of mulberry seedlings when exposed to Cd alone ( $P < 0.05$ ), with greater increases in females than in hermaphrodites and males. The MDA contents of females, hermaphrodites and males exposed to Cd increased by 79.2%, 40.5%, and 31.7%, respectively, compared to the control plants. On the other hand, the combined N + Cd treatment decreased MDA in all three kinds of mulberry seedlings. The MDA contents of females, hermaphrodites and males exposed to the combined N + Cd treatment increased by 29.3%, 24.3% and 11.5%, respectively, compared to the control plants. There was a significant interaction effect between Cd and N on MDA ( $P < 0.05$ ). SOD activities of plants exposed to Cd significantly decreased compared to those of control plants ( $P < 0.05$ ), while POD and CAT activities significantly increased ( $P < 0.05$ ). Compared to Cd treatments, the combined N + Cd treatment decreased POD and CAT activities in all three kinds of mulberry seedlings. There was a significant interaction effect between Cd and N on SOD, POD and CAT activities

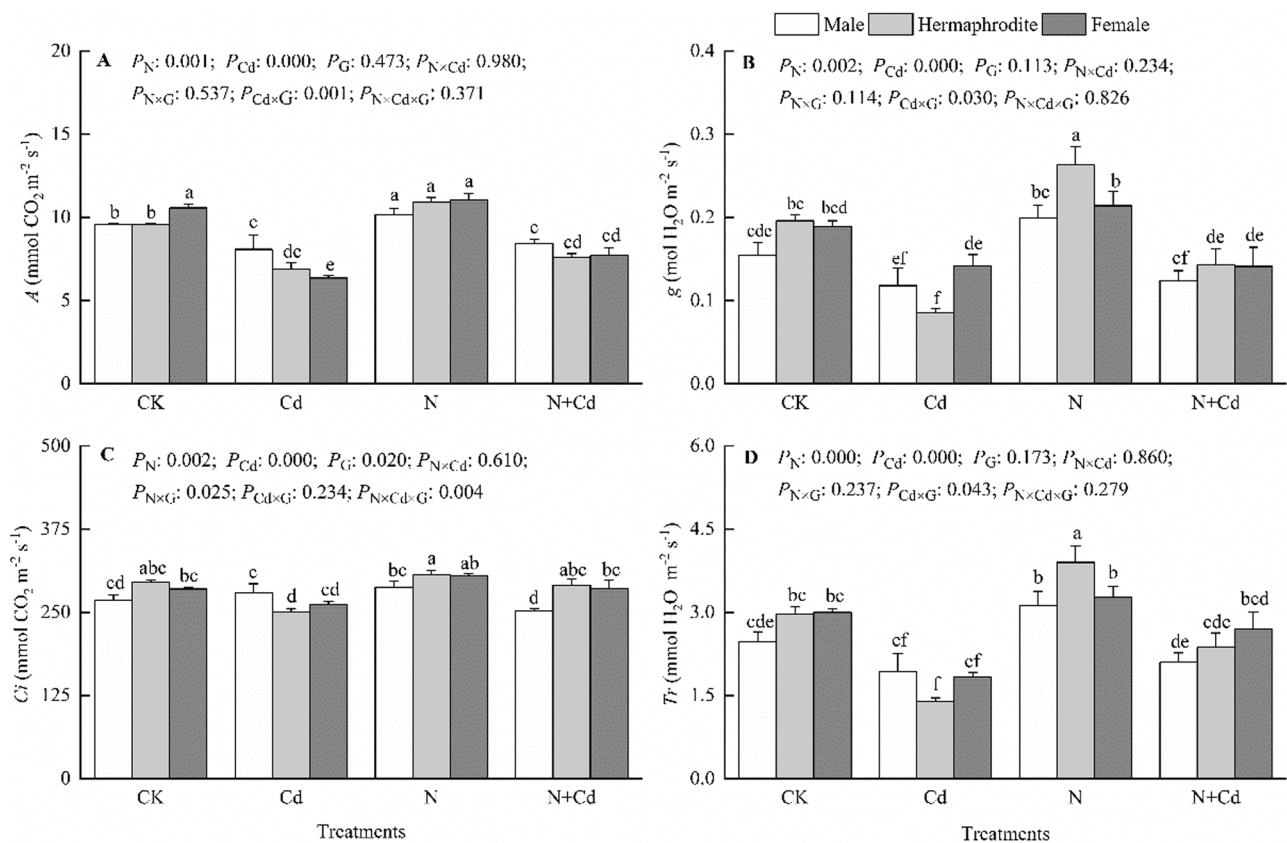
( $P < 0.05$ ).

### 3.4. Proline and non-protein thiol contents

The proline content of plants exposed to Cd increased significantly ( $P < 0.05$ ), and the increase in females and hermaphrodites was greater than that in males (Fig. 4). Furthermore, when plants were exposed to the combined N + Cd treatment, the proline contents of females, hermaphrodites and males increased by 81.0%, 80.5% and 28.8%, respectively, compared to control plants. Cd treatment also increased the content of non-protein thiols ( $P < 0.05$ ) (Fig. 4). Compared to the control plants, the non-protein thiol contents of females, hermaphrodites and males exposed to Cd alone increased by 129.7%, 181.6%, and 272.5%, respectively. When plants were exposed to the combined treatment, the non-protein thiol contents of females, hermaphrodites and males increased by 41.0%, 48.2%, and 65.7%, respectively. There was a significant interaction effect between Cd and N on the proline and non-protein thiol contents ( $P < 0.05$ ).

### 3.5. Biomass and root architecture

Cd treatment significantly reduced biomass in all tissues (roots, stems and leaves) of the three kinds of mulberry seedlings, with greater decreases observed in female and hermaphrodite tissues (Fig. 5). N treatment also significantly reduced root biomass in males, hermaphrodites and females ( $P < 0.05$ ). The combined N + Cd treatment decreased the total biomass of males, hermaphrodites, and females by 43.9%, 70.8% and 69.2%, respectively, compared to control plants. Females and hermaphrodites under the combined treatment allocated



**Fig. 2.** Effects of cadmium (Cd), nitrogen addition (N) and their combination on net photosynthesis rate (A) (A), stomatal conductance (g) (B), intercellular CO<sub>2</sub> concentration (Ci) (C), and transpiration (Tr) (D) in *Morus alba* males, hermaphrodites and females. CK, control plants. Different letters represent statistically significant differences between treatments (mean  $\pm$  SE,  $n = 4$ ) at  $P < 0.05$  according to least significant difference (LSD) multiple range tests.  $P_N$ , N effect;  $P_{Cd}$ , Cd effect;  $P_G$ , sex effect;  $P_{N \times Cd}$ , the interactive effect of N and Cd;  $P_{N \times G}$ , the interactive effect of N and sex;  $P_{Cd \times G}$ , the interactive effect of Cd and sex;  $P_{N \times Cd \times G}$ , the interactive effect of N, Cd and sex.

more biomass to leaves than to stems, in contrast to plants under a single Cd or N treatment (Fig. 6). The ANOVA analysis showed that there was a significant interactive effect between Cd and N on total biomass, root biomass and stem biomass ( $P < 0.05$ ).

The root phenotypic traits of the three sexes clearly altered by the Cd treatment (Fig. 7 and Fig. S1), and there were significant sex-specific differences. Compared with the control plants, the total root length, total root surface, total root volume and root tip numbers of females decreased more than those of hermaphrodites and males under the Cd treatment. The root phenotypic traits under the combined Cd and N treatment had similar changes as those of the Cd treatment. The ANOVA analysis showed that there was no significant interactive effect between Cd and N on phenotype traits, except for the total root surface ( $P < 0.05$ ).

### 3.6. Cd accumulation, allocation and translocation

Mulberry grown under the Cd treatment resulted in the highest Cd content in roots (Table 1). Sex-specific differences were also observed in Cd accumulation and allocation. Females accumulated more Cd in all organs than males and hermaphrodites, while there were no significant sex-specific differences in the control treatment. Compared to the Cd treatment, the combined treatment significantly increased the Cd content in all organs of females ( $P < 0.05$ ) but had no effect on Cd in any hermaphrodite organ ( $P > 0.05$ ). The ANOVA analysis showed that there was a significant interactive effect between Cd and N on Cd accumulation in roots, stems and leaves ( $P < 0.001$ ). Total Cd<sub>BCF</sub> in females under the combined treatment was highest (18.63%) among the three kinds of mulberries. Compared with the single Cd treatment, the

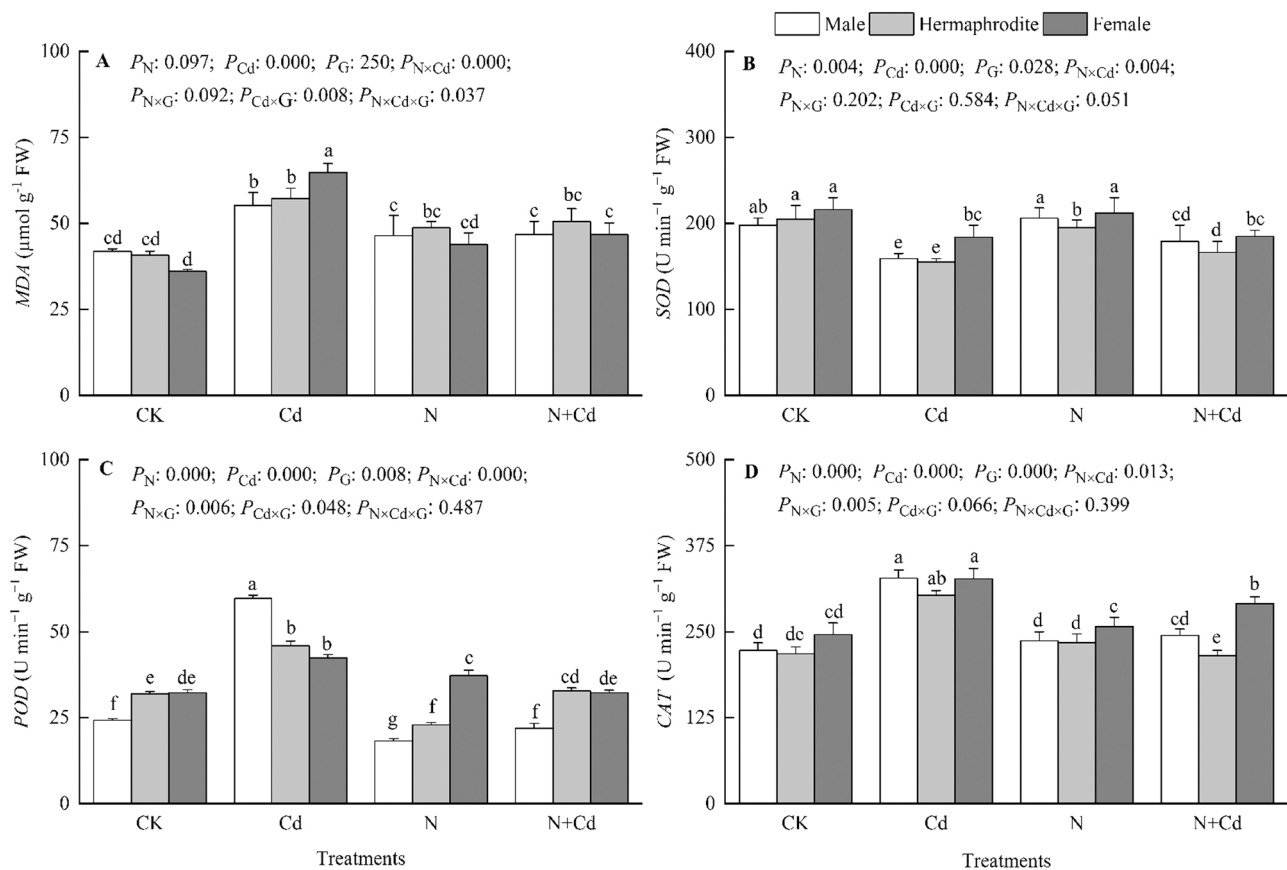
stem and leaf Cd<sub>BCF</sub> of females exposed to the combined N + Cd treatment increased by 34.48% and 64.29%, respectively, and the root Cd<sub>BCF</sub> of males exposed to the combined treatment increased by 29.93%. The leaf Cd<sub>BCF</sub> of males and hermaphrodites was significantly smaller than that of females (Table 2). However, there was no significant change in any organ Cd<sub>BCF</sub> in hermaphrodites. Under the single Cd treatment and the combined N + Cd treatment, the Cd<sub>TF</sub> value of females and hermaphrodites was significantly higher than that of males.

## 4. Discussion

### 4.1. Sexual differences in physiological and physicochemical responses to N and Cd

Cd has been reported to have negative effects on the physiology, antioxidant systems, and Cd accumulation in plants (Deng et al., 2017; El Rasafi et al., 2020; Gill et al., 2012). In line with these previous reports, our results also showed that all three kinds of mulberry seedlings responded to Cd with impaired photosynthetic apparatus and certain detoxification mechanisms with significant sex-specific differences.

As indicated by the decreases of *Fv/Fm* and *YII* in the three kinds of mulberry seedlings, Cd damaged the photosynthetic system II reaction centers of mulberry, and caused adverse effects on chlorophyll fluorescence (Gill et al., 2012; Szopiński et al., 2019). Therefore, inhibited carbon assimilation resulted from different stomatal limitation and PSII damage in mulberry trees, and resulted in the sexual response differences shown by the smaller decreases in *Fv/Fm*, *YII*, A, and g in males than those of females and hermaphrodites under Cd stress alone. Another evidence was the changes of mulberry defense system. Excess



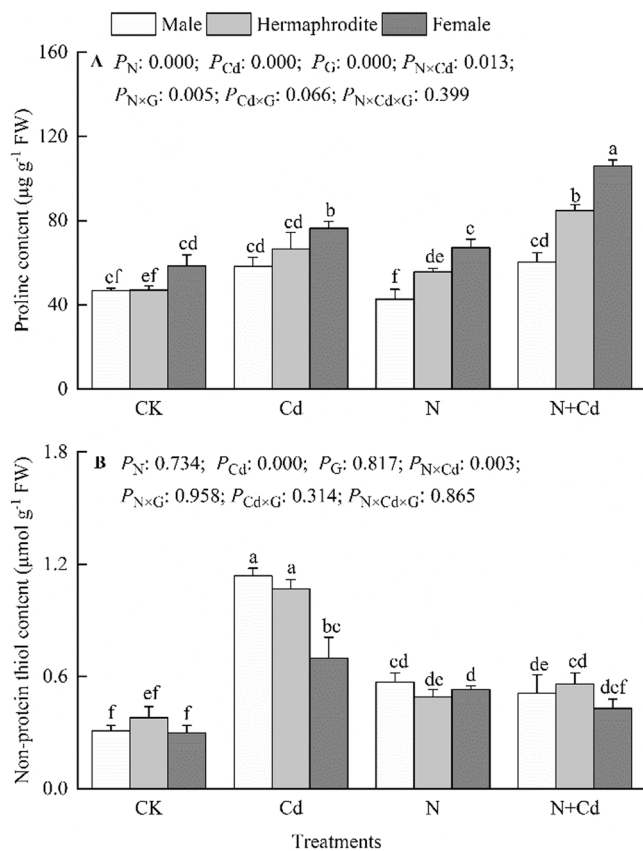
**Fig. 3.** Effects of cadmium (Cd), nitrogen addition (N) and their combination on the malondialdehyde (MDA) content (A), superoxide dismutase (SOD) activity (B), peroxidase (POD) activity (C), and catalase (CAT) activity (D) in *Morus alba* males, hermaphrodites and females. CK, control plants. Different letters represent statistically significant differences between the treatments (mean  $\pm$  SE,  $n = 4$ ) at  $P < 0.05$  according to least significant difference (LSD) multiple range tests.  $P_N$ , N effect;  $P_{Cd}$ , Cd effect;  $P_G$ , sex effect;  $P_{N \times Cd}$ , the interactive effect of N and Cd;  $P_{N \times G}$ , the interactive effect of N and sex;  $P_{Cd \times G}$ , the interactive effect of Cd and sex;  $P_{N \times Cd \times G}$ , the interactive effect of N, Cd and sex.

Cd can cause oxidative stress in plants by producing reactive oxygen species, which damage the antioxidant enzyme system and induce cellular damage (El Rasafi et al., 2020). As indicators related to plant injury, MDA, SOD, POD, CAT, proline, and non-protein thiols could be affected by Cd accumulation in plants (Zeng et al., 2009; Deng et al., 2017; Rady et al., 2019). Our results found that the antioxidant enzyme system of mulberry seedlings was disordered by Cd stress, while the function of antioxidant enzyme system in females and hermaphrodites was recovered partly by N deposition, which clearly showed the complicated defense system in mulberry with sex-specific responses. Cd caused damage to the cellular membrane system of mulberry leaves indicated by increased MDA contents (Sharma and Dietz, 2009; Cuyper et al., 2016). Females with highest MDA contents suffered more from peroxidation than did males, similarly to poplars' responses to Cd stress (Chen et al., 2011). It implied that males experienced fewer negative effects induced by Cd than did females and hermaphrodites. Melnikova et al. (2017) previously showed females are more sensitive to unfavorable environments than males. This sexual difference responses to unfavorable environment were conducive to meeting the resource demands of different sexes.

Simulated N deposition influenced the negative effects of Cd and sex-specific differences, which was reflected by no significant changes in *Fv/Fm*, *YII*, *qN*, and *SPAD* under the combined N + Cd treatments compared to the control treatments in three sexes of mulberry seedlings. This suggested that N deposition could alleviate the damage of Cd to PSII. However, N addition did not completely alleviate the inhibiting effect of Cd on the photosynthesis of mulberry since the experiment lasted for three months. Zhong et al. (2019) has also found N addition

may increase the sensitivity of plants to other stresses, and the influence of N addition on other environmental stress effects depends on the properties of the intensity and duration of the stress exposure. Interestingly, the A values of males exposed to the single Cd treatment were significantly higher than those of the females and hermaphrodites, but the differences were eliminated when the plants were exposed to the combined N + Cd treatment. Thus, N addition could alleviate Cd toxicity to PSII and lessen sex-specific differences in carbon assimilation caused by Cd stress.

In addition, although the changes of antioxidant enzyme activities in this study revealed that the antioxidant enzyme system was not sufficient to overcome excessive active oxygen-mediated damage caused by excess Cd in leaves, we also found the effect of N + Cd interaction on antioxidant system indicating the alleviated Cd damage and the sex-specific difference. Apart from this, increased proline and non-protein thiol contents would be beneficial for antioxidant defense system to alleviate the adverse effects of Cd stress. N-stimulated increases in proline and non-protein thiol contents under Cd were probably due to the improved N nutritional status and active nitrogen assimilation, especially due to the increased biosynthesis of amino acids (Meng et al., 2016). Another reason may be that N addition affected soil characteristics which changed the forms of heavy metals in the soil and the adsorption and desorption of heavy metals, thereby affecting the absorption and accumulation of Cd in plants (Yang et al., 2020). Thus, it is evident that mulberry has the ability to avoid severe damage from Cd exposure, and N could improve Cd uptake and transport, which is not contradictory to the fact that N could enhance the Cd tolerance of mulberry. Plants could adopt several strategies to avoid or mitigate Cd



**Fig. 4.** Effects of cadmium (Cd), nitrogen addition (N) and their combination on the proline content (A) and non-protein thiol content (B) in *Morus alba* males, hermaphrodites and females. CK, control plants. Different letters represent statistically significant differences between treatments (mean  $\pm$  SE,  $n = 4$ ) at  $P < 0.05$  according to least significant difference (LSD) multiple range tests.  $P_N$ , N effect;  $P_{Cd}$ , Cd effect;  $P_G$ , sex effect;  $P_{N \times Cd}$ , the interactive effect of N and Cd;  $P_{N \times G}$ , the interactive effect of N and sex;  $P_{Cd \times G}$ , the interactive effect of Cd and sex;  $P_{N \times Cd \times G}$ , the interactive effect of N, Cd and sex.

damages, for example, the retention of Cd by cell wall and vacuolar compartmentation (Shahid et al., 2017), alleviating reactive oxygen species (ROS) damages by regulating the activities of antioxidative enzymes (Yang et al., 2020), or storing photoassimilates as hexoses or complex sugars to minimize Cd uptake (Wang et al., 2019a). N deposition could enhance these strategies (Yang et al., 2020).

#### 4.2. Sexual differences in biomass responses to N and Cd

As reported in other study (Szopiński et al., 2019), Cd decreased the biomass in mulberry. Females were more sensitive to Cd stress as females showed more decreases in biomass in all tissues under Cd stress. Interestingly, Cd or N addition significantly decreased the root biomass allocation and increased the aboveground biomass allocation, especially stem biomass. This can be explained by the theory that plants would allocate more biomass to the organs that acquire the most limiting resources (Mccarthy and Enquist, 2007). In order to ensure the growth of aboveground parts, mulberry seedlings increased resource allocation to aboveground parts when roots were Cd-stressed, especially those of females and hermaphrodites. Biomass partitioning reflected plant tolerance to environmental stress. The results were perhaps because the Cd accumulation in roots influenced the uptake and transportation of nutrient from roots to the shoots (Bari et al., 2019; Khaliq et al., 2019) which ultimately damaged the plant growth.

Although exogenous N did not affect the total biomass except for hermaphrodites, the interaction Cd+N altered the biomass allocation of

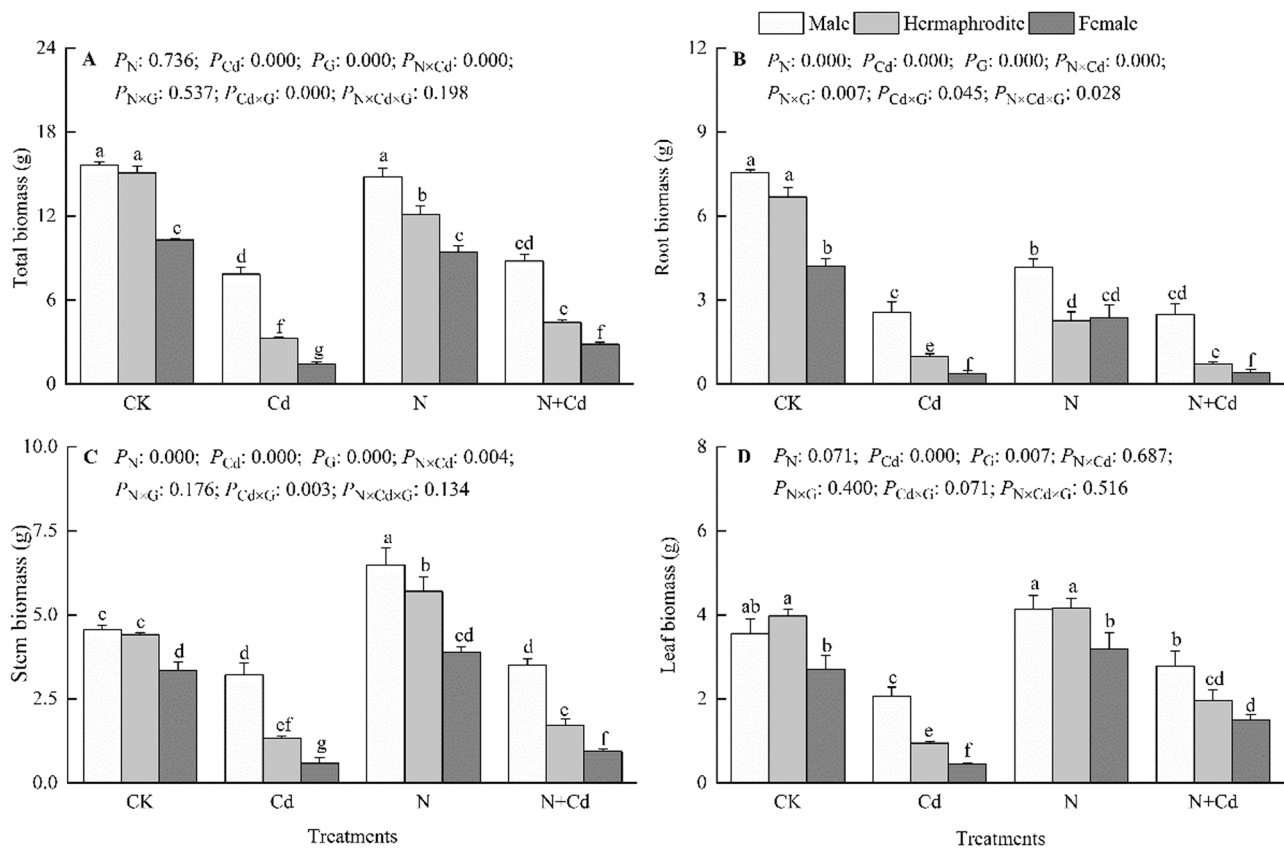
mulberry seedlings, showing the decreased root biomass allocation and the increased aboveground biomass allocation. Similar effects can be found in other studies. For example, Bronson et al. (2021) described that N fertilizer didn't improve biomass in *Parthenium argentatum* in arid areas due to the high nitrate content in the soil profile which hindered the N fertilizer absorption. He et al. (2020) reported that N fertilizer didn't induce higher total biomass but altered biomass allocation in 2- to 3-year triploid *Populus tomentosa* plantation on sandy loam soils on the North China Plain because N fertilizer uptake in plants were affected by soil moisture content. These results revealed that N fertilizer effect on plant biomass was influenced by other factors. Although there was a certain degree of recovery from the changes of leaf biomass when N was added under Cd treatment, root biomass was drastically affected by Cd and this effect was not reverted by the addition of N. Therefore, exogenous N was not enough to rescue plants from Cd toxicity, independently of the sex. Our study showed that N fertilizers improved Cd accumulation in mulberry but did not promote the root biomass and root characteristics, thus, more biomass was further allocated to aboveground parts.

#### 4.3. Relationship of Cd accumulation and root architecture

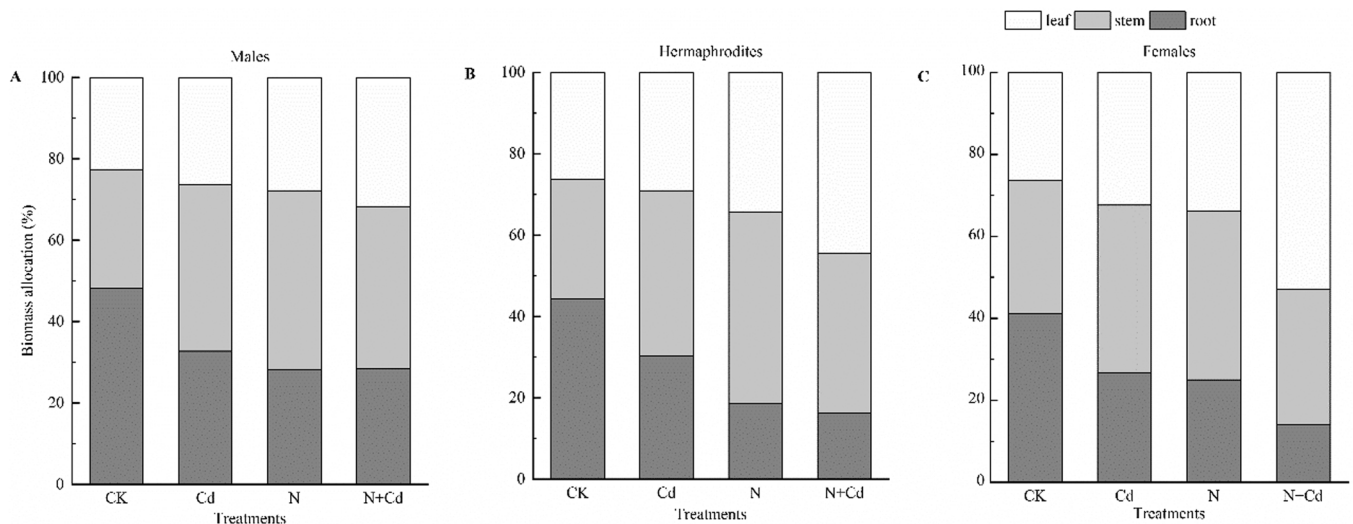
In this study, the roots of the three kinds of mulberry seedlings became shorter and thicker, indicating that mulberry had a strategy of decreasing the uptake of Cd by shortening root length, and reducing the number of fine roots and root tips (Lux et al., 2010). Therefore, root growth inhibition is one of the earliest observable symptoms of Cd toxicity. Root characteristics have a major influence on metal uptake (Lux et al., 2010). Root structure, root size, root surface area (Lux et al., 2010) and root hairs (Zheng et al., 2011) may play important roles in the absorption of Cd. Cd ions in soil enter the root through the epidermis (Sidhu et al., 2019), and root tips are the first tissues that make contact with bulk soil. Root hairs provide a large surface area for the absorption of Cd ions (Zheng et al., 2011). We found that root characteristics changes in females were greater than in males. This is related to the higher  $Cd_{TF}$  and leaf  $Cd_{BCF}$  in females. Leaves were more susceptible to Cd toxicity than roots, so females reduced more than males in the root length, surface area and root tip number in order to decrease Cd absorption and translocation from roots to shoots. In addition, the total  $Cd_{BCF}$  and root tip number of hermaphrodites were significantly lower, and their root length and root tip number decreased less than those of females and males, indicating that hermaphrodite roots might have a stronger ability to prevent Cd from entering the root system. This also demonstrated that the root system plays a vital role in the responses to adjust Cd stress (Bari et al., 2019) since Cd accumulation in plants may depend on Cd translocation from roots to shoots (Song et al., 2019).

However, the changes in root architecture affected by Cd were not reverted by N addition. This may be related to the Cd uptake and accumulation. Our results revealed that most of Cd was restricted in mulberry roots. The Cd content in the roots of the three types of mulberry seedlings was 10–40 times higher than that in the aboveground parts. In general, the maximum portion of Cd absorbed by roots entered the root cells, but only a small portion was translocated to the shoots and leaves (Song et al., 2019). The intracellular Cd distribution facilitated Cd detoxification in the roots and hence promoted tolerance against Cd toxicity. The internal distribution of Cd within different parts of plants was relevant to the bioavailability of heavy metals, translocation abilities from roots to shoots and plant tolerance patterns (Jiang et al., 2019), which resulted in different Cd accumulation rates observed in different plant tissues. N addition increased Cd uptake in the three types of mulberry seedlings, which was reflected in increased  $Cd_{BCF}$ . In fact, a large number of studies have recognized that N fertilizer had a positive effect on Cd accumulation in plants (Leite and Monteiro, 2019; Du et al., 2009; Yang et al., 2020). One reason for the increased Cd accumulation in plants was that N improved Cd exchange capacity and bio-available Cd contents in soils, thus releasing sufficient Cd to the roots of plants





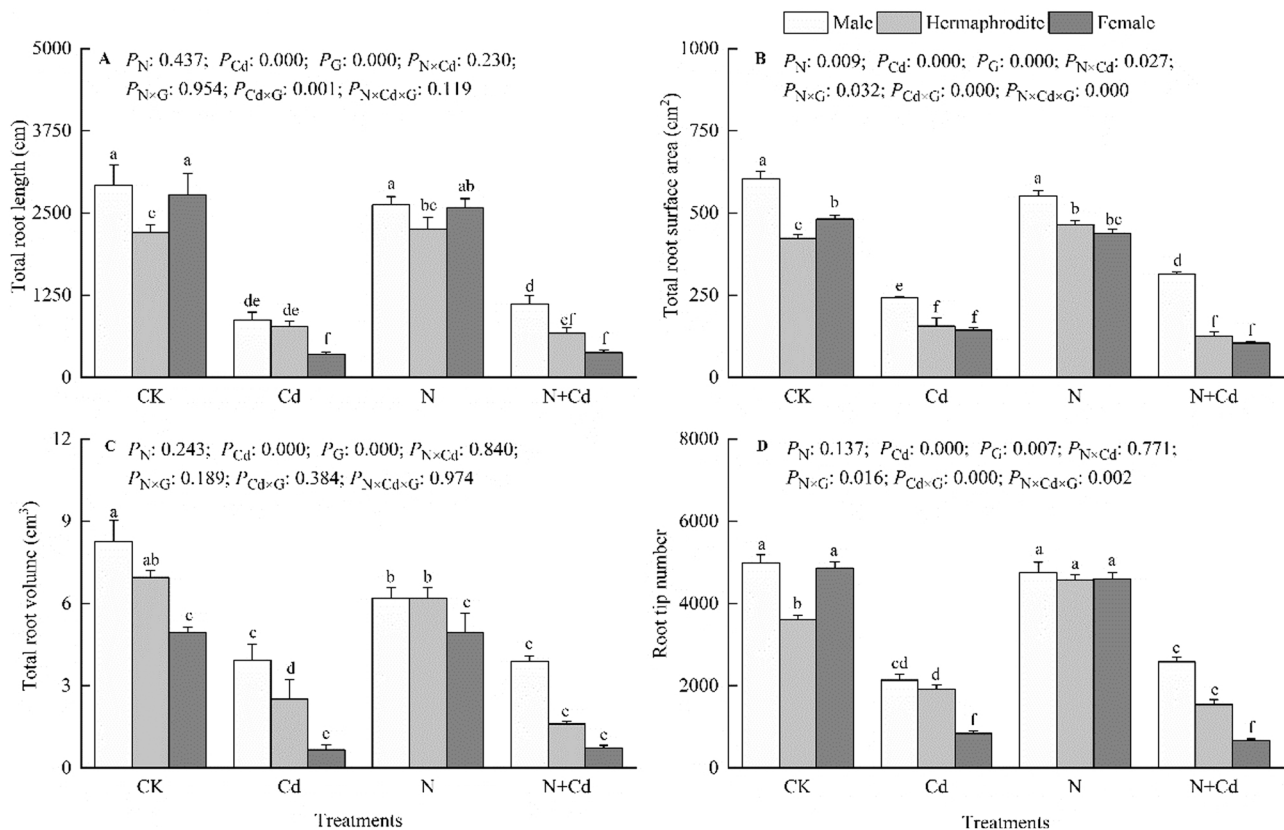
**Fig. 5.** Effects of cadmium (Cd), nitrogen addition (N) and their combination on total biomass (A), root biomass (B), stem biomass (C), and leaf biomass (D) in *Morus alba* males, hermaphrodites and females. CK, control plants. Different letters represent statistically significant differences between treatments (mean  $\pm$  SE,  $n = 4$ ) at  $P < 0.05$  according to least significant difference (LSD) multiple range tests.  $P_N$ , N effect;  $P_{Cd}$ , Cd effect;  $P_G$ , sex effect;  $P_{N \times Cd}$ , the interactive effect of N and Cd;  $P_{N \times G}$ , the interactive effect of N and sex;  $P_{Cd \times G}$ , the interactive effect of Cd and sex;  $P_{N \times Cd \times G}$ , the interactive effect of N, Cd and sex.



**Fig. 6.** Effects of cadmium (Cd), nitrogen addition (N) and their combination on biomass allocation in *Morus alba* males (A), hermaphrodites (B) and females (C) ( $n = 4$ ). CK, control plants.

(Zhang et al., 2018). Furthermore, N increased Cd uptake by regulating NO-induced divalent cation transporters, which played important roles in Cd absorption from the rhizosphere to the roots, as well as in the transport from roots to shoots (Qin et al., 2020). In addition, N regulates cell wall isolation, chelation, and oxidative damage to enhance Cd tolerance, thus also increasing Cd uptake and accumulation in plants

(Chai et al., 2018; Zhang et al., 2020b; Zhen et al., 2021). These results indicate that root characteristics played a crucial part in resisting Cd stress. Therefore, further other factors such as root exudates, which may also affect sex-specific responses to Cd in mulberry (Bali et al., 2020), should be taken into consideration.



**Fig. 7.** Effects of cadmium (Cd), nitrogen addition (N) and their combination on the total root length (A), total root surface area (B), total root volume (C), and root tip number (D) in *Morus alba* males, hermaphrodites and females. CK, control plants. Different letters represent statistically significant differences between treatments (mean ± SE, n = 4) at P < 0.05 according to least significant difference (LSD) multiple range tests. P<sub>N</sub>, N effect; P<sub>Cd</sub>, Cd effect; P<sub>G</sub>, sex effect; P<sub>N×Cd</sub>, the interactive effect of N and Cd; P<sub>N×G</sub>, the interactive effect of N and sex; P<sub>Cd×G</sub>, the interactive effect of Cd and sex; P<sub>N×Cd×G</sub>, the interactive effect of N, Cd and sex.

**Table 1**

Effects of cadmium (Cd), nitrogen addition (N) and their combination on the Cd content of *Morus alba* males, hermaphrodites and females.

N (kg N ha <sup>-1</sup> ·yr <sup>-1</sup> )	Cd (mg·kg <sup>-1</sup> dry soil)	Sex	Total Cd content (mg·kg <sup>-1</sup> )	Root Cd content (mg·kg <sup>-1</sup> )	Stem Cd content (mg·kg <sup>-1</sup> )	Leaf Cd content (mg·kg <sup>-1</sup> )
0	0	M	10.91 ± 1.21e	3.97 ± 0.25e	4.57 ± 0.88fg	2.37 ± 0.31ef
0	0	H	13.36 ± 1.53e	7.82 ± 0.32e	3.52 ± 0.24fg	2.03 ± 0.04f
0	0	F	15.34 ± 1.57e	7.63 ± 0.11e	5.22 ± 1.91fg	2.49 ± 0.63ef
0	100	M	1010.67 ± 121.62c	976.72 ± 22.04c	30.06 ± 0.47de	3.90 ± 0.04de
0	100	H	580.88 ± 32.94d	543.82 ± 62.24d	31.08 ± 2.14e	5.99 ± 0.25cd
0	100	F	1096.97 ± 71.15b	1009.94 ± 71.10b	75.09 ± 1.40b	11.94 ± 0.71b
90	0	M	13.09 ± 2.46e	2.86 ± 0.21e	6.09 ± 0.31fg	4.15 ± 0.15de
90	0	H	7.59 ± 0.97e	4.43 ± 0.31e	1.59 ± 0.09g	1.57 ± 0.13f
90	0	F	16.15 ± 2.15e	7.31 ± 0.54e	6.63 ± 0.71f	2.20 ± 0.15ef
90	100	M	1299.63 ± 136.17b	1269.34 ± 38.38b	25.54 ± 2.13e	4.75 ± 0.54d
90	100	H	649.22 ± 36.96d	605.48 ± 36.60d	36.78 ± 1.20c	6.94 ± 0.73c
90	100	F	1601.88 ± 64.61a	1481.18 ± 66.91a	100.65 ± 14.07a	20.05 ± 1.64a
		P <sub>N</sub>	0.000	0.002	0.000	0.000
		P <sub>Cd</sub>	0.000	0.000	0.000	0.000
		P <sub>G</sub>	0.000	0.000	0.000	0.000
		P <sub>N×Cd</sub>	0.000	0.002	0.001	0.000
		P <sub>N×G</sub>	0.000	0.003	0.000	0.000
		P <sub>Cd×G</sub>	0.000	0.000	0.000	0.000
		P <sub>N×Cd×G</sub>	0.036	0.002	0.000	0.001

Different letters represent statistically significant differences between treatments (mean ± SE, n = 4) at P < 0.05 according to least significant difference (LSD) multiple range tests. P<sub>N</sub>, N effect; P<sub>Cd</sub>, Cd effect; P<sub>G</sub>, sex effect; P<sub>N×Cd</sub>, the interactive effect of N and Cd; P<sub>N×G</sub>, the interactive effect of N and sex; P<sub>Cd×G</sub>, the interactive effect of Cd and sex; P<sub>N×Cd×G</sub>, the interactive effect of N, Cd and sex.

**5. Conclusions**

Cd toxicity caused changes with sex-specific differences in growth, chlorophyll fluorescence parameters, gas exchange, root architecture and biomass in male, female and hermaphrodite seedlings of mulberry. In response, mulberry plants deployed detoxification mechanisms to

avoid or tolerate the toxic effects of Cd. Simulated N deposition alleviated the negative effects of Cd on leaves and decreased sex-specific differences in the three kinds of mulberry seedlings, but N fertilizer did not affect the total biomass. The N-stimulated increasing in proline and non-protein thiol contents might play a crucial role in resisting the damage caused by Cd stress, and the three kinds of mulberry seedlings

**Table 2**Effects of cadmium (Cd), nitrogen addition (N) and their combination on Cd<sub>BCF</sub> and Cd<sub>TF</sub> in *Morus alba* males, hermaphrodites and females.

N (kg N ha <sup>-1</sup> yr <sup>-1</sup> )	Cd (mg kg <sup>-1</sup> dry soil)	Sex	Total Cd <sub>BCF</sub>	Root Cd <sub>BCF</sub>	Stem Cd <sub>BCF</sub>	Leaf Cd <sub>BCF</sub>	Cd <sub>TF</sub>
0	0	M	/	/	/	/	/
0	0	H	/	/	/	/	/
0	0	F	/	/	/	/	/
0	100	M	11.75 ± 0.25c	11.36 ± 0.26b	0.35 ± 0.01d	0.05 ± 0.01c	0.035 ± 0.003c
0	100	H	6.75 ± 0.38d	6.32 ± 0.39c	0.36 ± 0.02 cd	0.07 ± 0.01c	0.068 ± 0.003b
0	100	F	12.76 ± 1.68 bc	11.74 ± 1.67b	0.87 ± 0.02b	0.14 ± 0.01b	0.086 ± 0.012a
90	0	M	/	/	/	/	/
90	0	H	/	/	/	/	/
90	0	F	/	/	/	/	/
90	100	M	15.11 ± 0.42b	14.76 ± 0.45a	0.30 ± 0.02d	0.06 ± 0.01c	0.024 ± 0.003c
90	100	H	7.55 ± 0.43d	7.04 ± 0.42c	0.43 ± 0.01c	0.08 ± 0.02c	0.072 ± 0.003ab
90	100	F	18.63 ± 0.75a	17.22 ± 0.77a	1.17 ± 0.05a	0.23 ± 0.02a	0.081 ± 0.006ab

Different letters represent statistically significant differences between treatments (mean ± SE, n = 4) at P &lt; 0.05 according to least significant difference (LSD)

multiple range tests. Cd<sub>BCF</sub>, Cd bioconcentration factor; Cd<sub>TF</sub>, Cd translocation factor; Total Cd<sub>BCF</sub> =  $\frac{\text{Cd content in plant roots, stems and leaves (mg}\cdot\text{kg}^{-1})}{\text{Cd content in the soil (mg}\cdot\text{kg}^{-1})}$ ;

Root Cd<sub>BCF</sub> =  $\frac{\text{Cd content in plant roots (mg}\cdot\text{kg}^{-1})}{\text{Cd content in the soil (mg}\cdot\text{kg}^{-1})}$ ; Stem Cd<sub>BCF</sub> =  $\frac{\text{Cd content in plant stems (mg}\cdot\text{kg}^{-1})}{\text{Cd content in the soil (mg}\cdot\text{kg}^{-1})}$ ; Leaf Cd<sub>BCF</sub> =  $\frac{\text{Cd content in plant leaves (mg}\cdot\text{kg}^{-1})}{\text{Cd content in the soil (mg}\cdot\text{kg}^{-1})}$ ; Cd<sub>TF</sub> =  $\frac{\text{Cd content in stems and leaves (mg}\cdot\text{kg}^{-1})}{\text{Cd content in roots (mg}\cdot\text{kg}^{-1})}$ .

had slightly different ways of improving Cd tolerance by N deposition. Sexual differences in Cd accumulation are correlated with root architecture. Thus, this study provides evidence for the use of mulberry to treat Cd-contaminated soils under N deposition, and further knowledge for selecting plants that have highest tolerance to Cd pollution. However, the mechanisms of sexual differences in responses to Cd in plants are not yet entirely resolved. Hence, the following study on Cd stress in mulberry can be recommended to identify the genes responsible for Cd tolerance and accumulation (El Rasafi et al., 2020). Furthermore, from the future prospective, more work can be carried out in the fields to find out the mechanism of sexual differences in soil microorganism and root exudates regarding the impact on Cd bioaccumulation and transformation.

### CRediT authorship contribution statement

Fei Yu: Writing – original draft. Lita Yi: Conceptualization. Xiaoyu Mao: Investigation. Qi Song: Formal analysis. Helena Korpelainen: Writing – review & editing. Meihua Liu: Writing – review & editing, Supervision.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113288.

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