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**Microstructural and physiological responses to cadmium stress under different
nitrogen levels in *Populus cathayana* females and males**

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Head title: Sexual differences in responses to Cd and N deficiency in poplar

Abstract Although an increasing attention has been paid on the relationships between heavy metal and nitrogen availability, the mechanism underlying adaptation to Cd stress in dioecious plants has been largely overlooked. This study examined Cd accumulation, translocation and allocation among tissues and cellular compartments in *Populus cathayana* females and males. Both leaf Cd accumulation and root-to-shoot Cd translocation were significantly greater in females than in males under a normal N supply, but they were reduced in females and enhanced in males under N deficiency. The genes related to Cd uptake and translocation, *HMA2*, *YSL2* and *ZIP2*, were strongly induced by Cd stress in female roots and in males under a normal N supply. Cd largely accumulated in the leaf blades of females and in the leaf veins of males under a normal N supply, while the contrary was true under N deficiency. Furthermore, Cd was mainly distributed in the leaf epidermis and spongy tissues of males, and in the leaf palisade tissues of females. N deficiency increased Cd allocation to the spongy tissues of female leaves and to the palisade tissues of males. In roots, Cd was preferentially distributed to the epidermis and cortices in both sexes, and also to the vascular tissues of females under a normal N supply but not under N deficiency. These results suggested that males possess better Cd tolerance compared to females, even under N deficiency, which is associated with their reduced root-to-shoot Cd translocation, specific Cd distribution in organic and/or cellular compartments, and enhanced antioxidation and ion homeostasis. Our study also provides new insights into engineering woody plants for phytoremediation.

Keywords: dioecy, sexual differences, Cd distribution, nitrogen level, sequestration.

Introduction

Cadmium (Cd) is a nonessential and highly toxic element for plants. Cd is not only harmful to plant growth and metabolism but it also threatens human health, as large amounts of Cd may enter the food chain (Godt et al. 2006; Li et al, 2018). Phytoremediation by plants has been proposed to be an effective biotechnological strategy to remediate Cd-contaminated soils (Castagna et al. 2013, Li et al. 2018). Plants have evolved a series of strategies for Cd detoxification and tolerance. Cd could be sequestered into cell walls and/or vacuoles, and it could induce antioxidant synthesis to alleviate oxidative stress (Peng et al. 2017, Zhang et al. 2018). Different plant species show have different tolerances to Cd and employ different mechanisms to reduce Cd toxicity, but there can be different detoxification mechanism engaged even by the same species among different genotypes (Meyer et al. 2015, 2016).

Poplars have been suggested as promising candidates for remediating heavy metal-polluted soils due to their high growth rates and low impact on the food chain (Iori et al. 2016). Thus, both from the wood production and phytoremediation point of view it is important to improve poplars' growth and tolerance under Cd stress. The use of nitrogen fertilizers has been recently suggested to be one of the most important practices to alleviate Cd toxicity in plants (Chen et al., 2011; Liu et al., 2017), while Cd toxicity

affects nitrogen absorption and metabolism (Erdal & Turk, 2016). Cd inhibits NO₃⁻ uptake and impairs nitrate homeostasis, resulting in a decrease in nitrate transport from roots to shoots (Mao et al. 2014). Some enzymes related to nitrogen metabolism, such as nitrate reductase, glutathione synthase and glutamate synthetase, are also affected by Cd stress (Sharma et al. 2010, Erdal & Turk, 2016). In turn, some nitrogen metabolites, such as proline, glutathione (GSH) and phytochelatins (PCs), facilitate Cd detoxification in plants (Sharma & Dietz, 2006). Therefore, changes in the nitrogen status of plants may affect the stress caused by Cd.

Previous studies have suggested that responses of nitrogen metabolism to Cd stress vary among plant species, even among genotypes within the same species (Liao et al. 2019), as observed, e.g., in *Medicago sativa* (Yang et al. 2019). Furthermore, recent studies have shown that dioecious plants, e.g., *Populus* species, display sexual differences in defense responses to abiotic stress, including Cd toxicity, males usually displaying a better tolerance compared to females (Li et al. 2016, Chen et al. 2017), but the mechanisms causing sexual differences in Cd tolerance are poorly known. We have previously found that *P. yunnanensis* females are more sensitive to Cd stress than males, but N deposition could mitigate Cd toxicity and decrease sexual differences (Chen et al., 2011). However, sex-specific responses to combinations of Cd and N availability and underlying mechanisms have not been elucidated in *P. cathayana*. Therefore, we investigated physiological and molecular mechanisms related to nitrogen status and cadmium toxicity in *P. cathayana* males and females in order to reveal potential sex-

specific response patterns and to test the efficacy of enhanced N availability to alleviate Cd stress.

Materials and Methods

Plant material and growth conditions

Cuttings of *P. cathayana* females and males were collected from 60 different trees sampled in 15 populations, containing 30 females and 30 males, in the riparian and valley flat habitats of the Qinghai Province, China. Annual temperature, mean annual rainfall and annual solar radiation in the area are 6.9 °C (maximum 38 °C, minimum - 20 °C), 335 mm and 4500 MJ m⁻², respectively (Zhao et al., 2009). Cuttings were rooted as described by Chen et al. (2015). The experimental design was with three factors (sex, N and Cd), i.e. two sexes (females, males), two Cd regimes (-Cd, +Cd) and two N levels (N deficiency, sufficient N). The seedlings were grown in a greenhouse at the Hangzhou Normal University. After one month, uniform cuttings were chosen and transplanted into plastic pots with a 10 kg mixture of sand, vermiculite and perlite (1:1:1). Every three days, 100 ml of nutrient solution and 100 ml of sterile distilled water were used for irrigation. The composition of the nutrient solution was as follows (μM): 500 μM KCl, 900 μM CaCl₂, 300 μM MgSO₄, 0.1 μM CuSO₄, 0.5 μM MnSO₄, 600 μM KH₂PO₄, 42 μM K₂HPO₄, 2000 μM NH₄NO₃, 25 μM Fe-EDTA, 10 μM H₃BO₃, 0.5 μM ZnSO₄, and 0.1 μM (NH₄)₆Mo₇O₂₄. The pH of the solution was adjusted to 6.0 using HCl. After

the seedlings had been growing in sandy pots for 30 d, uniform cuttings were subjected to Cd and N treatments for 120 d. In the N treatment, the seedlings were irrigated with a complete 2000 μM NH_4NO_3 (+N+Cd) or NH_4NO_3 -free nutrient solution (-N+Cd), and the final NH_4NO_3 level reached 200 mg N kg^{-1} soil. In the Cd treatment, $\text{CdCl}_2 \cdot 2.5 \text{ H}_2\text{O}$ of 100 μM was applied to the sandy pots every day during the first 40 d, and the final Cd level reached 50 mg $\text{CdCl}_2 \cdot 2.5 \text{ H}_2\text{O}$ kg^{-1} dry soil.

Growth measurements

The plants were collected after the end of the experiments. Samples of roots, leaves and stems were first oven-dried at 105 °C for 1 h and then dried at 70 °C until a constant mass was reached, after which the dry mass (DM) was estimated.

Gas exchange measurements and estimation of photosynthetic pigments

The fourth fully expanded leaves were chosen to measure the net photosynthesis rate, stomatal conductance and transpiration rate using the LI-6400 photosynthesis measuring system (Li-Cor, Inc., Lincoln, NE, USA) at 08:00-11:30 h. The measuring conditions were as follows: 1500 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photosynthetic photon flux density, 25 °C leaf temperature, 70% air humidity and 400 $\mu\text{mol mol}^{-1}$ ambient CO_2 concentration. In addition, the leaves were extracted in 80% cooled acetone (v/v) in the dark until the leaves changed their color to white. Chlorophyll and carotenoid concentrations were

measured from measurements of solution absorbances at 470, 646 and 663 nm, and calculated according to Chen et al. (2011).

Determination of reactive oxygen species and enzyme activities

The reactive oxygen species, malondialdehyde (MDA) and enzyme activities were measured according to the method by Chen et al. (2011). For H₂O₂, c. 0.2 g of leaves and roots were ground with liquid nitrogen and then with 5% trichloroacetic acid, followed by Chen et al. (2011). Briefly, 0.2 ml of clear supernatant was mixed with 1 ml 20% TiCl₄ (v/v, dissolved in HCl) and 0.2 ml ammonia, and then centrifugated at 5000 g for 10 min. The precipitation was dissolved in 1.5 M H₂SO₄, and measured at 410 nm. For O₂⁻ determination, the leaves and roots were finely ground with the extraction mixture (50 mM Na₂HPO₄-NaH₂PO₄, pH 7.8) and then centrifugated at 12000 g for 10 min. A volume of 0.5 ml of supernatant was mixed with 0.1 ml hydroxylamine hydrochloride (10 mM). The reaction was conducted at 25 °C for 30 min. O₂⁻ levels were measured colorimetrically at 540 nm after adding 1 ml of 0.2% N-(1-naphthyl)-ethylene diamine and 1 ml of 1% sulfanilamide. For MDA, the leaves and roots were ground with 10% trichloroacetic acid and centrifuged at 12000 g for 10 min. Then, 0.5 ml of clear supernatant was let to react with 2 ml thiobarbituric acid (0.6%) in a boiling water bath for 15 min. MDA was measured colorimetrically at 450, 320 and 600 nm, and calculated as follows: $C \text{ (nM)} = 6.45 (OD_{532} - OD_{600}) - 0.56 \times OD_{450}$.

The activities of peroxidase (POD), superoxide dismutase (SOD), glutathione (GR) and

catalase (CAT) were measured as described by Chen et al. (2011). Proteins were measured using the Bradford method.

Determination of Cd and nutrient elements

Dried leaves and roots were finely ground and dissolved in 3:1 (v/v) of HNO₃ and HClO₄. Total Cd and nutrient elements were measured with ICP-MS (inductively coupled plasma mass spectrometer Agilent 7500a, Agilent Technologies). The translocation factor (T_f) was defined as the ability for root-to-shoot Cd translocation and calculated as the ratio of Cd concentration in shoots to roots (Shi et al. 2010).

Microscopic imaging of Cd, P and S localization in roots, leaf blades and veins

Leaves and roots were washed carefully with deionized H₂O. Subsequently, the samples were cut into sections and dried with a vacuum freeze dryer for 100 h. The sample surfaces were gold-plated with vacuum sputtering. Photographs were taken under a scanning electron microscope (Zeiss Sigma 500, German) at 3 kV. The line scan on the sample surface was conducted with an energy-dispersive x-ray (EDX) (EDAX ELEMENT, America) at 10 kV voltage. The spectra of Cd, P and S on the surface was analyzed with the SuperQuant program (EDAX).

Quantitative PCR analyses of gene expression related to Cd uptake and transport

177

178 Approximately 0.1 g of roots and leaves was finely ground with liquid nitrogen. Total
179 RNA was isolated using a RNA extraction kit (TaKaRa MiniBEST Plant RNA
180 Extraction Kit, TaKaRa, Otsu, Japan). The first cDNA strand was synthesized using
181 PrimeScript reverse transcription (RT) reagent kits (Takara) according to instructions.
182 Quantitative RT polymerase chain reactions (qRT-PCR) were conducted with One Step
183 TB GreenTM PrimeScriptTM RT-PCR kits in a 25 µl reaction system with pairs of
184 specific primers (He et al. 2013) (Table S1).

185

186 Heavy metal ATPase 2 and 4 (HMA2 and HMA4) proteins facilitate the root-to-shoot
187 translocation of Cd (Li et al. 2018). Metallothionein-like protein (MTP1) and yellow
188 stripe-like protein (YSL2) are responsive for Cd transportation into the vacuoles
189 (Ricachenevsky et al. 2013). The zinc transporter 2 and 6.2 (ZIP2 and ZIP6.2) regulate
190 the Cd translocation into the cell cytosol of roots (Ma et al. 2014). These genes were
191 analyzed in this study. The primers were similar as those in He et al. (2015). *TUB4.1*
192 was used as housekeeping genes. The relative expression of specific genes was
193 calculated according to Liu et al. (2017).

194

195 *Analysis of Fourier transform infrared spectroscopy (FTIR)*

196

197 The leaves and roots were washed carefully with deionized H₂O. Subsequently, the
198 samples were dried with a vacuum freeze dryer for 100 h. The freeze-dried powder of

leaves and roots was pressed against the diamond crystal of an attenuated total reflectance device and the infra-red spectra were determined with FTIR spectrometer Nicolet iS5. The scanning range was 400-4000 cm^{-1} wavenumber.

As shown in Table S2, the differential spectral peaks are at 1651 cm^{-1} for C-N vibration from protein, at 1419 cm^{-1} for vibration of COO^- from pectin, at 1317 cm^{-1} for C-O vibration from cellulose, at 1151 cm^{-1} for vibration of C-C and C-O stretch from carbohydrates (such as soluble sugar, cellulose and hemicellulose), at 1235 cm^{-1} for C=O vibration from xylans and lignin, at 1071 cm^{-1} for C-O from cellulose and hemicellulose, at 1743 cm^{-1} for vibration of C=O from esterified pectin, and at 1111 cm^{-1} for C-C or C-O vibration from pectin.

Statistical analysis

Differences among means within treatments were separated by Duncan's test using the SPSS software (version 22.0) with three-way analyses when $P < 0.05$. Data were checked for the normality before analyses. The principal component analysis (PCA) was computed by the command procomp () in R (<http://www.R-project.org/>) according to Luo et al. (2019).

Results

Sexual differences in leaf gas exchange characteristics, pigments and biomass

Cd stress, N deficiency and the combined stress reduced A in both sexes, especially in females (Table 1). The stomatal conductance (g_s) was not affected by stress in females, but it was significantly reduced in males under the combined treatment (Table 1). All stresses increased the intercellular CO₂ concentration (C_i) in both females and males (Table 1). The chlorophyll concentrations of a, b, a+b, and carotenoids were reduced in females, while no change was found in males under any stress. Additionally, N deficiency did not affect these chlorophylls relative to a normal N supply in either sex exposed to Cd stress.

In females, the dry mass of leaves, stems and roots, and total plant dry mass decreased under nitrogen deficiency and Cd stress, and more seriously under the combined treatment (Table 2). In males, these values decreased under nitrogen deficiency and combined stress, but Cd stress did not affect leaf dry mass when compared to Cd-free conditions. Under control conditions (high N, no Cd), females showed a higher leaf and root dry mass, and total biomass, but a lower stem dry mass when compared to males. In addition, N deficiency significantly increased the ratio of root to shoot irrespective of the Cd treatment in both sexes.

Sexual differences in oxidative stress and antioxidants

243 N deficiency, Cd stress and the combined treatment increased H_2O_2 and O_2^- of leaves,
244 and MDA, H_2O_2 and O_2^- of roots in females compared with control plants. Males
245 showed higher H_2O_2 in roots and leaves under all stresses when compared to controls.
246 O_2^- in male roots and leaves was not affected by Cd stress, but its concentration
247 increased in male roots under N deficiency, and in leaves under N deficiency and the
248 combined stress. Furthermore, females showed higher MDA in roots and O_2^- in leaves
249 under all stress conditions compared to males. There was no significant difference
250 between sexes in leaf MDA under N deficiency, in leaf H_2O_2 under Cd stress, or in root
251 H_2O_2 under the combined treatment. In contrast, males had higher H_2O_2 in leaves under
252 the combined treatment and in roots under N deficiency alone.

253

254 In males, Cd stress, N deficiency and the combined treatment significantly increased
255 SOD in roots and leaves, as well as CAT in roots, but it did not affect POD and GR in
256 roots (Fig. 2). In females, POD and GR of roots were reduced under all stresses, while
257 CAT of roots and leaves, as well as POD and GR of leaves increased under Cd stress.
258 The combined stress increased CAT and SOD in leaves and SOD in roots in females.
259 When compared to females, males had higher POD in leaves, CAT and POD in roots,
260 and SOD in roots and leaves under the combined treatment (Fig. 2). However, there
261 was no significant difference between sexes in GR of roots under N deficiency and Cd
262 stress, and in POD of roots and leaves under the combined stress. CAT in leaves under
263 N deficiency and the combined stress, and SOD in roots under the combined stress were
264 higher in females than in males.

Sexual differences in Cd accumulation

Elevated Cd exposure strongly increased Cd accumulation in leaves, roots, stem wood and bark in both sexes (Fig. 3). Males had higher Cd levels in roots, but lower Cd in leaves compared to females under a normal N supply. Cd levels in barks and stem woods were not different between females and males under a normal N supply and Cd stress (+N+Cd). When compared to a normal N supply, N deficiency combined with Cd stress strongly promoted Cd accumulation in leaves, roots and woods of males, while females had lower Cd levels in leaves, barks and woods. Cd in roots of females and in barks of males showed no differences between N deficiency and a normal N supply under Cd stress (Fig. 3). Additionally, males had higher Cd in leaves, roots, woods and barks than females under the combined stress. The translocation factor T_f was significantly higher in females than in males under Cd stress, but no significant difference was detected under the combined treatment (Fig. S1).

Sexual differences in Cd allocation within organs

The energy-dispersive x-ray (EDX) and scanning electron microscope (TEM) were used to explore Cd distribution in the cross-sections of leaf blades, leaf veins and roots. Females had a stronger Cd signal in leaf vein cross-sections compared to males under control conditions (Fig. 4). N deficiency reduced Cd allocation into vein cross-sections

in both sexes, but more strongly in males. Furthermore, in females, a large amount of Cd distributed into epidermal and cortical tissues, as well as into vascular tissues, especially into phloem under a normal N supply. In contrast, N deficiency increased Cd allocation to the leaf vein phloem, epidermis and cortices, especially to the upper epidermis of leaf veins in females (Fig. 4). In males, more Cd was allocated to the cortices of the abaxial veins of leaves, as well as to vascular tissues, especially in xylems. N deficiency combined with Cd stress induced considerable Cd allocation to the epidermis and cortices of the abaxial veins, as well as to xylems in males.

In leaf blade cross-sections, the Cd signal intensity was higher in females than in males under a normal N supply (Fig. 5). N deficiency increased the intensity of Cd signals throughout the male leaf blades, but reduced them in females. Specifically, more Cd distributed into the mesophyll of females, while males had strong Cd signals in the upper epidermis and mesophyll under a normal N supply. N deficiency increased the Cd distribution in epidermal tissues in females, especially in the upper epidermis (Fig. 5). In contrast, more Cd was allocated to the mesophylls and lower epidermal tissues of male leaf blades under the combined treatment. Furthermore, Cd signals largely distributed in female mesophylls, especially in the palisade tissues, while males had high Cd in the spongy tissues under a normal N supply (Fig. 5). N deficiency largely increased the proportion of Cd allocation to the spongy tissues of leaf blades in both sexes when compared to normal N supply conditions.

In roots, males had slightly higher Cd signals throughout the cross-sections when compared to females under a normal N supply (Fig. 6). N deficiency increased Cd accumulation in the cross-sections of males, but reduced Cd signals in females. Under a normal N supply, Cd signals were strongest in the epidermal, cortical and vascular tissues of females, while males had more Cd in epidermal and cortical tissues. N deficiency induced more Cd allocation to epidermal and cortical tissues, and less to vascular tissue in females (Fig. 6). However, more Cd signals were detected in the epidermal, cortical and vascular tissues of males under the combined treatment.

Sexual differences in S and P allocation among tissues

The allocation of P and S were also studied in the cross-sections of roots, leaf veins and blades by the application of EDX analysis and SEX imaging. In leaf blades, females had a higher P to S ratio, while the contrary was true for males under a normal N supply (Fig. S2). N deficiency increased the proportion of S in both sexes. Cd stress increased S in leaf blade cross-sections relative to P in both sexes, especially in females treated without a N supply and in males treated with a normal N supply (Fig. 7).

In leaf vein cross-sections, the ratio of S to P signal was higher in epidermal and cortical tissues of females when compared to males but not in vascular tissues (Fig. S2). Males had stronger S signals than P signals under a normal N supply, and S and P were uniformly distributed throughout the cross-sections of leaf veins under both N levels

(N deficiency, normal N supply). In females, N deficiency induced P allocation to vascular tissues, but the proportions of P and S were similar in epidermal and cortical tissues. Cd stress significantly increased S throughout leaf vein cross-sections in both sexes, especially in males (Fig. 7). Moreover, P and S were mainly distributed in the vascular tissues of males under N deficiency. The proportion of S relative to P in roots was highest in N-sufficient females and N-deficient males (Fig. S2). Cd stress increased P in female roots under a normal N supply and P of males under N deficiency relative to Cd-free controls (Fig. 7).

FTIR spectra of roots and leaves

To investigate the effect of Cd on the chemical fingerprint of the molecular composition of the cell wall, we measured the absorption spectra peaks of leaves and roots. In this study, PCA analysis was performed to study the original absorbance data. In leaves, PC1 and PC2 accounted for 92% and 2% of the variation, respectively (Fig. 8; Table S3). Peaks at 1651, 1419, 1317 cm^{-1} were the most vital contributors to PC1, whereas peaks 1111 and 1541 cm^{-1} were the key factors contributing to PC2. In roots, PC1 and PC2 accounted for 95% and 2% of the variation, respectively. In the PCA plot of roots, peaks 1419, 1317, 1111 and 1157 cm^{-1} were key factors contributing to PC1, whereas peaks at 3360 cm^{-1} and 2915 cm^{-1} were main contributors to PC2 (Fig. 8; Table S4). The PCA results indicated that males showed more significant changes in the chemical composition of leaves and roots compared with females under both N supply levels, but

especially under a normal N supply, reflecting the absorption by the groups related to lignin, cellulose, hemicellulose and pectin in roots and leaves.

PCA of physiological responses

To uncover the main factors participating in the adaptive responses of females and males to Cd stress and N deficiency, PCA was performed using traits related to photosynthesis, growth, element concentrations, oxidative stress and antioxidative capacity (Fig. 9; Table S5). PC1 and PC2 accounted for 40% and 19% of the variation, respectively. Shoot dry mass, stem mass, net photosynthesis rate and leaf H₂O₂ levels were key factors contributing to PC1, while root H₂O₂ levels, *g_s* and transpiration rates were the three most important factors contributing to PC2. The PCA separated females from males in responses to Cd stress and N deficiency.

Transcript levels of genes involved in Cd translocation and uptake

We analyzed the genes related to Cd translocation and tolerance in roots. Cd induced the expression of *HMA2* and *HMA4* genes in female roots, whereas in male roots Cd stress downregulated *HMA2i* but did not affect *HMA4* expression compared to the controls (Fig. 10). N deficiency did not affect the expressions of *HMA2* and *HMA4* in female roots, but it upregulated the expression of *HMA2* under Cd stress compared to controls. Cd stress induced *MTP1* gene expression in female roots, especially under N

deficiency, while the transcription of *MTP1* in male roots was strongly induced by Cd stress but down-regulated under the combined stress. The expression of *YSL2* in roots was induced in females by Cd stress but inhibited in males, while it was upregulated by the combined stress in both sexes. The transcription levels of *ZIP2* and *ZIP6.2* genes in male roots and *ZIP2* of female roots were upregulated by Cd stress irrespective of the N status, while the expression of *ZIP6.2* gene in female roots was not affected by Cd stress but downregulated under the combined stress.

Discussion

Sexually different physiological tolerance to Cd stress and N deficiency

Cadmium interferes with plant growth and metabolism, but the toxic effects of Cd differ among plant species (Baliardini et al. 2015, He et al. 2015). In this study, Cd stress significantly reduced A and leaf dry mass in females but not in males under a normal N supply (Tables 1, 2). This result is consistent with previous studies (Chen et al. 2011; Chen et al. 2016). Noticeably, the damage on photosynthesis and biomass accumulation was smaller in males than in females under the combined stress (Table 2). Interestingly, we also found that males showed no significant symptoms of Cd toxicity irrespective of the N supply, while clear Cd toxicity symptoms were found in the abaxial leaves of females under a normal N supply but not under N deficiency (Fig. S3). It could be inferred that males have a far stronger Cd tolerance compared to females, especially

under a normal N supply (Fig. S3). Yet, Cd toxicity symptoms of females did not differ much between N deficiency and a normal N supply, which could be explained by the reduced Cd translocation to leaves (Fig. S1). Our results appeared different from those of Chen et al (2011), who proposed that N deposition decreases differences in Cd sensitivity in *Populus yunnanensis* females and males. However, the N supply level and species were different in the study by Chen et al (2011). They used normal and higher N applications, while we used N deficiency and a normal N supply. It seems that the N availability (N deficiency, normal N and high N) differently affects the responses of females and males to Cd stress.

Cd accumulation in leaves disrupts photosynthesis (Fei et al. 2018). Thus, the inhibition of Cd translocation to the shoots is probably an effective approach to enhance Cd tolerance (Daud et al. 2015, Fei et al. 2018). Cd uptake, root-to-shoot translocation and accumulation in leaves were largely induced under a normal N supply, while N deficiency reduced Cd accumulation in the leaves of females (Fig. 3). This is in accordance with previous studies (Chang et al., 2013, Hu et al., 2013). The lower Cd accumulation in female leaves under N deficiency is probably a self-protecting strategy to cope with Cd toxicity, as described by Zhang et al (2019), who suggested that lower Cd accumulation in *Populus* leaves might be a self-protecting strategy to prevent severe oxidative damage due to a decreased stress tolerance under N deficiency. Cheng et al (2017) also suggested that ammonium-based fertilizers enhance Cd accumulation in *Carpobrotus rossii*. However, this is not the case in *P. cathayana* males, since more Cd

accumulated in leaves under N deficiency than under a normal N supply (Fig. 3). Similarly, Konotop et al (2012) suggested that a nitrogen application decreases Cd uptake and improves Cd tolerance in soybean seedlings. Perilli et al (2010) have also proposed that cadmium concentrations of wheat are influenced by the nitrogen level, seedling age and soil type. The greater Cd accumulation in male leaves under N deficiency is probably correlated with N sensitivity, since males have a lower resource consumption and stronger tolerance to N deficiency compared to females. Moreover, the capacity for antioxidation defense was significantly elevated by N deficiency in males (Fig. 2). Evidently, males and females employ different mechanisms to cope with Cd toxicity, especially under N deficiency.

Changed nutrient allocation and oxidative-antioxidation homeostasis in females and males under N deficiency highlight the physiological regulation mechanism of Cd

Populus species are characterized by dioecy, which is usually associated with sexual dimorphism. Females generally allocate more resources to reproduction and males often increase investment in defense (Juvany & Munné-Bosch, 2015). Sulfur compounds, such as GSH, PCs and metallothionein, act as antioxidants or chelators involved in plant tolerance to heavy metals (Cobbett & Goldsbrough 2002, Li et al. 2019). Therefore, P and S allocation to roots, leaf blades and veins exposed to Cd stress were analyzed in this study (Fig. 7). Females had higher proportions of P relative to S in leaves under control conditions, especially in leaf blades, which is consistent with

their higher reproductive investment (Graff et al. 2013). In contrast, the proportion of
 S relative to P was higher in males under control conditions, which is consistent with
 their stronger tolerance to stress (He et al. 2013). S and P allocations were closely
 correlated with Cd throughout cross-sections of leaf blades and veins in both sexes,
 especially in males under a normal N supply and in females under N deficiency (Figs.
 4-7). Cd detoxification by S has been previously reported (Sarwar et al. 2010, Chen et
 al. 2015). In addition, He et al (2015) found in poplars that S was significantly
 correlated with Cd and it increased GSH synthesis through the overexpression of
 bacterial γ -glutamylcysteine synthetase facilitated by Cd detoxification and enhanced
 Cd tolerance. Hence, the stronger tolerance of males is probably attributable to the high
 proportion of S in males under Cd stress. It should be noted that N deficiency increased
 the proportion of P in males under Cd stress. P is involved in PC biosynthesis, Cd is
 transported into vacuoles by Cd/PC complexes, and Cd is sequestered into cell walls
 through an association with phosphates (Parrotta et al. 2015). Hence, the increase in P
 induced by N deficiency probably plays an important role in the Cd tolerance of males.

Cd toxicity is often accompanied with a ROS burst, which causes the disruption of
 redox homeostasis, followed by oxidative damage on plant cells (Rui et al. 2016, Gupta
 et al. 2017). The present study found that Cd induces oxidative damage more seriously
 in females than in males, especially by O_2^- in leaves and by MDA in roots (Fig. 1). ROS
 act to stimulate an early defective response (Liu et al. 2018a, b), and elevated H_2O_2
 levels detected in male and female leaves and roots are early signals of adaptive

responses to stress, including the induction of antioxidants. Antioxidants, such as GR, SOD, POD and CAT, are regarded as main enzymatic antioxidants scavenging the detrimental effects of ROS in plants (Schutzendubel et al. 2001). Cd stress reduced CAT, POD and GR activities in female leaves and roots but had little effect on males compared to controls (Fig. 2). Furthermore, although N deficiency promoted Cd accumulation in male leaves but reduced that in females, females were still more sensitive to Cd stress compared to males.

Sexually different Cd sequestration and accumulation among tissues under N deficiency highlight the mechanism of Cd tolerance

It is worth noting that N deficiency reduced Cd uptake and/or translocation in females but increased those in males under Cd stress. The extensive Cd accumulation in shoots without toxicity symptoms is similar as what happens in hyperaccumulator plants, which are characterized by a high capacity of root-to-shoot translocation (Lu et al. 2013). We found that genes related to Cd uptake and translocation, such as *HMA2* and *HMA4*, *YSL2* and *ZIP2*, were strongly induced by Cd stress in female roots under a normal N supply (Fig. 10). In male roots, the expression of *HMA2*, *YSL2* and *ZIP6* genes were significantly induced by N deficiency. Additionally, strong sequestration into the cell walls of male roots under a normal N supply reduced Cd accumulation in shoots (Fig. 8).

Males showed a better Cd tolerance when compared to females under both N supply levels, although N deficiency promoted Cd uptake and/or translocation from roots to shoots in males (Fig. 3, Fig. S1). This is consistent with observations on the hyperaccumulating ecotype of *Sedum alfredii* Hance, in which Cd accumulates in shoots without toxicity symptoms (Tian et al. 2017). Successful Cd detoxification probably requires effective sequestration of Cd into organs (Lu et al. 2013), and effective Cd sequestration among tissues and cellular compartments, as observed in *P. cathayana* males, is probably accomplished as a permanent Cd storage (Tian et al. 2009). We found that Cd largely accumulates in male bark, especially under N deficiency (Fig. 3), which is consistent with previous investigations (He et al. 2013). Epidermal Cd increased in leaf veins and blades, primarily in the upper leaf epidermis and spongy tissues of males, especially under N deficiency, perhaps protecting leaf mesophylls and guard cells against Cd toxicity (Figs. 4-5). Furthermore, successful Cd detoxification probably need effective sequestration in appropriate cellular compartments to accomplish the status of permanent storage (Tian et al. 2013). The cell walls and vacuoles are suggested to effectively sequester Cd and reduce cytosolic Cd levels in plants (Peng et al. 2017, Zhang et al. 2018). In this study, Cd stress induced the expression of *HMA2*, *HMA4* and *MTP1* genes in male leaves, especially the expression of *MTP1* under N deficiency (Fig. S4). Additionally, the higher ratio between males and females at 1419, 1317, 1111 and 1157 cm⁻¹ of FTIR suggested that higher pectin and lignin levels probably contributed to Cd detoxification in males.

Conclusions

The present study suggests that one of the primary factors responsible for a greater Cd allocation to leaves and sensitivity in females is ineffective Cd sequestration in organs and/or cellular compartments (Fig. 11). Moreover, the more extensive root-to-shoot translocation of Cd and the weaker Cd detoxification in females also led to their greater sensitivity to Cd toxicity, irrespectively of the N supply. Although N deficiency reduced the Cd root-to-shoot translocation in females and elevated that in males, males had a better Cd tolerance compared to females under Cd stress. It follows that it may be important to modify artificially the soil N status depending on the Cd tolerance of *P. cathayana* females and males. In all, our investigation provides new insights into efforts aiming to engineer woody plants for phytoremediation.

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

531

532 **Supporting Information** Additional supporting information and references can be
533 found in the supplementary information.

534

535 **References**

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Table 1. Net photosynthesis rate (A), stomatal conductance (g_s), transpiration (E), chlorophyll a (Chl a), Chl b and Chl (a+b), and carotenoid (Car) in leaves of *Populus cathayana* females and males, as affected by cadmium stress, N deficiency and their combination.

Sex	Treatment	A	g_s	E	Chl a	Chl b	Chl (a+b)	Car
Female	+N-Cd (Control)	17.37±0.80c	0.223±0.026c	4.00±0.48d	1.79±0.031a	0.410±0.031a	2.20±0.074a	0.897±0.029a
	-N-Cd	10.11±1.07ef	0.223±0.035c	3.80±0.44d	0.851±0.13c	0.260±0.091c	1.11±0.23c	0.587±0.017cd
	+N+Cd	12.69±1.66d	0.194±0.024c	3.52±0.31d	1.42±0.13b	0.394±0.14ab	1.82±0.26b	0.492±0.093d
	-N+Cd	9.56±0.64f	0.203±0.029c	3.98±0.37d	0.910±0.097c	0.248±0.035c	1.16±0.18c	0.509±0.012d
Male	+N-Cd (Control)	20.96±1.40a	0.368±0.083ab	5.31±0.87ab	1.73±0.076a	0.419±0.11a	2.15±0.12a	0.769±0.01ab
	-N-Cd	12.26±0.26d	0.326±0.052b	4.97±0.52bc	1.34±0.048b	0.304±0.50bc	1.65±0.88b	0.694±0.010bc
	+N+Cd	19.01±1.40b	0.409±0.06a	6.07±0.70a	1.71±0.21a	0.400±0.063ab	2.11±0.23a	0.741±0.20b
	-N+Cd	11.49±1.53de	0.231±0.068c	4.10±0.89cd	1.40±0.12b	0.359±0.029ab	1.76±0.15b	0.754±0.13b
P_s		***	***	***	ns	ns	ns	***
P_{cd}		***	ns	*	**	ns	**	**
P_n		***	**	***	***	***	***	***
$P_{s \times cd}$		ns	ns	ns	ns	ns	ns	***
$P_{s \times n}$		***	**	ns	*	ns	ns	ns
$P_{cd \times n}$		**	ns	***	**	ns	*	**
$P_{s \times cd \times n}$		ns	ns	ns	nsns	ns	ns	ns

F_s , sex effect; P_{cd} , Cd effect; P_n , N effect; $P_{s \times cd}$, the interaction effect of sex and Cd. $P_{s \times n}$, the interaction effect of sex and N; $P_{cd \times n}$, the interaction effect of Cd and N; $F_{s \times cd \times n}$, the interaction effect of sex, Cd and N. Different letters on the bars indicate significant differences between the treatments ($P < 0.05$, Duncan's test). The significance values of the three-way analysis of variance are shown as follows: ns, not significant; * 0.01 $< P \leq 0.05$; ** 0.001 $< P \leq 0.01$; *** $P \leq 0.001$.

Table 2. The dry mass of leaves, stems, roots and total biomass, and the root: shoot ratio in *Populus cathayana* females and males, as affected by cadmium stress, N deficiency and their combination.

Sex	Treatment	Leaf mass (g)	Stem mass (g)	Root mass (g)	Total mass (g)	Root : Shoot
Female	+N-Cd (Control)	17.01 ± 0.93a	16.76 ± 0.77b	8.33 ± 0.31a	42.10 ± 0.87a	0.247 ± 0.014c
	-N-Cd	7.14 ± 0.84c	4.66 ± 1.06de	3.52 ± 0.42e	16.29 ± 1.62d	0.385 ± 0.074b
	+N+Cd	11.20 ± 1.54b	13.95 ± 1.04c	6.11 ± 0.58b	31.26 ± 1.99b	0.243 ± 0.022c
	-N+Cd	4.04 ± 0.94d	3.83 ± 0.67f	4.49 ± 0.63cd	11.39 ± 1.45e	0.510 ± 0.087a
Male	+N-Cd (Control)	8.11 ± 0.24c	18.13 ± 1.32a	4.81 ± 0.83c	31.05 ± 1.52b	0.174 ± 0.019c
	-N-Cd	2.90 ± 1.01de	5.74 ± 0.93d	2.37 ± 0.093f	11.41 ± 0.67e	0.265 ± 0.03c
	+N+Cd	7.03 ± 0.84c	14.88 ± 0.45c	3.96 ± 0.083de	25.86 ± 0.76c	0.181 ± 0.0086c
	-N+Cd	2.06 ± 0.26d	3.58 ± 0.66f	2.34 ± 0.37f	7.98 ± 0.34f	0.424 ± 0.11b
P_s		***	*	***	***	*
P_{cd}		***	***	***	***	**
P_n		***	***	***	***	***
$P_{s \times cd}$		***	ns	**	***	ns
$P_{s \times n}$		***	ns	**	***	ns
$P_{cd \times n}$		*	*	**	***	ns
$P_{cd \times m \times n}$		ns	ns	ns	*	ns

P_s , sex effect; P_{cd} , Cd effect; P_n , N effect; $P_{s \times cd}$, the interaction effect of sex and Cd. $P_{s \times n}$, the interaction effect of sex and N; $P_{cd \times n}$, the interaction effect of Cd and N; $P_{s \times cd \times n}$, the interaction effect of sex, Cd and N. Different letters on the bars indicate significant differences between the treatments ($P < 0.05$, Duncan's test). Values are expressed as means ± SE (n = 6). The significance values of the three-way analysis of variance are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

Figure legends

Figure 1 The concentrations of malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-) in the leaves and roots of *Populus cathayana* females and males, as affected by cadmium stress, N deficiency and their combination. P_s , sex effect; P_{cd} , Cd effect; P_n , N effect; $P_{s \times cd}$, the interaction effect of sex and Cd. $P_{s \times n}$, the interaction effect of sex and N; $P_{cd \times n}$, the interaction effect of Cd and N; $F_{s \times cd \times n}$, the interaction effect of sex, Cd and N. Different letters on the bars indicate significant differences between the treatments. Values are expressed as means $\pm$ SD ($n = 4$). The significance values of the three-way analysis of variance are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

Figure 2 The activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and glutathione reductase (GR) in the leaves and roots of *Populus cathayana* females and males exposed to cadmium stress, N deficiency and their combination. P_s , sex effect; P_{cd} , Cd effect; P_n , N effect; $P_{s \times cd}$, the interaction effect of sex and Cd. $P_{s \times n}$, the interaction effect of sex and N; $P_{cd \times n}$, the interaction effect of Cd and N; $F_{s \times cd \times n}$, the interaction effect of sex, Cd and N. Different letters on the bars indicate significant differences between the treatments. Values are expressed as means $\pm$ SD ($n = 4$). The significance values of the three-way analysis of variance are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

1 **Figure 3** Cd accumulation in the leaves, roots, wood and bark of *Populus cathayana*
2 females and males exposed to cadmium stress, N deficiency and their combination. P_s ,
3 sex effect; P_{cd} , Cd effect; P_n , N effect; $P_{s \times cd}$, the interaction effect of sex and Cd. $P_{s \times n}$,
4 the interaction effect of sex and N; $P_{cd \times n}$, the interaction effect of Cd and N; $F_{s \times cd \times n}$, the
5 interaction effect of sex, Cd and N. Different letters on the bars indicate significant
6 differences between the treatments. Values are expressed as means $\pm$ SD (n = 4). The
7 significance values of the three-way analysis of variance are shown as follows: ns, not
8 significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

9

10 **Figure 4** Cd distribution in the leaf vein cross-sections of *Populus cathayana* females
11 and males exposed to cadmium stress, N deficiency and their combination as
12 determined by energy-dispersive x-ray analysis and scanning electron microscope
13 imaging.

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15 **Figure 5** Cd distribution in the leaf blade cross-sections of *Populus cathayana* females
16 and males exposed to cadmium stress, N deficiency and their combination as
17 determined by energy-dispersive x-ray analysis and scanning electron microscope
18 imaging.

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20 **Figure 6** Cd distribution in the root cross-sections of *Populus cathayana* females and
21 males exposed to cadmium stress, N deficiency and their combination as determined
22 by energy-dispersive x-ray analysis and scanning electron microscope imaging.

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2 **Figure 7** Cd distributions in the root, leaf blade and vein cross-sections of *Populus*
3 *cathayana* females and males exposed to cadmium stress, N deficiency and their
4 combination as determined by energy-dispersive x-ray analysis and scanning electron
5 microscope imaging.

6

7 **Figure 8** FTIR spectra and the corresponding principle component analysis (PCA) plot
8 of leaves and roots of *Populus cathayana* females and males exposed to cadmium stress,
9 N deficiency and their combination. The average spectrum of leaves and roots was
10 plotted ($n = 4$). PCA was conducted with the data of selected peaks separately for leaves
11 and roots (Supplementary Table 1).

12

13 **Figure 9** Principal component analysis (PCA) plots of oxidants, antioxidants,
14 photosynthesis parameters, pigments and biomass in the leaves and roots of *Populus*
15 *cathayana* females and males exposed to cadmium stress, N deficiency and their
16 combination. PCA was performed using the data presented in Tables 1-2 and Figs 1-2.

17

18 **Figure 10** Effects of cadmium, nitrogen deficiency and their combination on the
19 expression of *heavy metal ATPase 2 and 4 (HMA2 and HMA4)*, *metallothionein-like*
20 *protein (MTPI)*, *yellow stripe-like protein (YSL2)*, *zinc transporter 2 and 6.2 (ZIP2 and*
21 *ZIP6.2)* genes in the roots of *Populus cathayana* females and males. Values are
22 expressed as means $\pm$ SD ($n = 4$). Different letters on the bars indicate significant

1 differences between the treatments ($P < 0.05$, Duncan's test).

2

3 **Figure 11** A schematic model for Cd accumulation, distribution and tolerance in
4 *Populus cathayana* females and males. CW, cell wall; PM, plasma membrane; ZIP2
5 and ZIP6.2, zinc/iron regulated transporter 2 and 6.2; ABCCs, ATP-binding cassette
6 transporter ; HMA2, P-type heavy metal ATPase 2; YSL2, yellow stripe-like2.

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1 **Figure 5**

1 **Figure 6**

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1 **Figure 7**

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1 **Figure 8**

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1 Figure 9

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1 **Figure 10**

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1 **Figure 11**

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