Journal of Experimental Botany, Vol. 62, No. 14, pp. 5037–5050, 2011
doi:10.1093/jxb/err203 Advance Access publication 21 July, 2011
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Nitrogen nutrient status induces sexual differences in responses to cadmium in *Populus yunnanensis*

Lianghua Chen[1], Ying Han[1,2], Hao Jiang[1], Helena Korpelainen[3] and Chunyang Li[1,*]

[1] Chengdu Institute of Biology, Chinese Academy of Sciences, PO Box 416, Chengdu 610041, China
[2] School of Life Sciences, Southwest University of Science and Technology, Mianyang 621010, China
[3] Department of Agricultural Sciences, PO Box 27, FI-00014, University of Helsinki, Finland

* To whom correspondence should be addressed. E-mail: licy@cib.ac.cn

Received 9 May 2011; Revised 30 May 2011; Accepted 31 May 2011

Abstract

Populus yunnanensis **was employed as a model species to detect sexual differences in growth, physiological, biochemical, and ultrastructural responses to cadmium (Cd) stress, nitrogen (N) deposition, and their combination. Compared with the control conditions, Cd decreased plant biomass, damaged the photosynthetic apparatus, visible as a decreased maximum efficiency of photosystem II (PSII; F_v/F_m) and effective quantum yield of PSII (Yield), depressed gas exchange capacity, and induced oxidative stress, visible as the disruption of antioxidative enzymes and accumulation of reactive oxygen species (ROS), in both sexes. On the other hand, Cd toxicity was mitigated by the recovery of gas exchange capacity, a decrease in ROS, and improvement of the redox imbalance in both sexes when N deposition was applied. However, males showed a higher gas exchange capacity, lower enzyme inhibition and ROS accumulation, stronger abilities to maintain cellular redox homeostasis, and a better maintenance of chloroplast ultrastructure than did females when exposed to Cd stress alone. Although males exhibited a higher Cd content in leaves than did females, males also accumulated higher levels of non-protein thiols (NP-SHs) and free amino acids (FAAs) for detoxification than did females. Sexual differences induced by Cd, visible, for example, in F_v/F_m, Yield, net photosynthesis rate (A), and stomatal conductance (g_s), decreased under N deposition, as no significant differences between the sexes existed in these parameters under the combined treatment. The results indicated that females are more sensitive to Cd stress and suffer more injuries than do males. Moreover, N deposition can mitigate Cd toxicity and decrease sexual differences in Cd sensitivity.**

Key words: Chlorophyll fluorescence, gas exchange, reactive oxygen species, redox homeostasis, ultrastructure.

Introduction

Cadmium (Cd), a non-essential element for plants, mainly derived from industrial processes, traffic pollution, phosphate fertilizers, and mineralization of rocks, occurs in soils at elevated levels as a result of fast developing agriculture and industry, especially in developing countries. Cd is not only negative for the growth and development of plants, but it also induces serious health concerns for humans and animals if excessive amounts enter food chains. Plants exposed to Cd suffer from a stress condition, visible as a series of toxic symptoms, such as chlorosis or necrosis, lowered gas exchange and growth rate, and alterations in water and nutrient status (Sandalio *et al.*, 2001; Metwally *et al.*, 2005; Küpper and Kochian, 2010). Cd also disturbs antioxidant systems and the redox balance, and causes generation of free radicals and reactive oxygen species (ROS). Low levels of ROS, such as $O_2\cdot^-$ and H_2O_2, could serve as signal molecules in the induction of defence genes against Cd toxicity, whereas overproduction of ROS would injure cellular biomolecules, such as nucleic acids, proteins, carbohydrates, and lipids (Mittler, 2002; Romero-Puertas *et al.*, 2002). Therefore, the capability for ROS scavenging is crucial for mitigating oxidative stress when plants are exposed to Cd. In addition, Cd detoxification plays a pivotal role in decreasing Cd toxicity when Cd enters plant cells.

An important mechanism is to form a complex of Cd with various substances, for example organic acids, amino acids, phytochelatins (PCs), and metallothioneins (Cobbett and Goldsbrough, 2002; Benavides *et al.*, 2005), and then to compartmentalize the ligand–metal complex, which can prevent the circulation of free Cd in the cytosol and will transport it into a limited area, such as the vacuole (Vögeli-Lange and Wagner, 1990).

Nitrogen (N) deposition, which is increasingly aggravated on a global scale due to the combustion of fossil fuels and use of N-containing fertilizers, not only affects plant growth and development, but also impacts species richness and biodiversity of ecosystems (Stevens *et al.*, 2004; Phoenix *et al.*, 2006). During recent years, more and more studies have paid attention to the relationship of N status and plant stress sensitivity, such as salinity (Ehlting *et al.*, 2007) and ozone (Utriainen and Holopainen, 2001). On the other hand, N metabolism of plants plays a central role in heavy metal responses. Cd sensitivity of plants is affected by both the N forms supplied (Hassan *et al.*, 2005; Xie *et al.*, 2009) and N availability (Panković *et al.*, 2000; Finkemeier *et al.*, 2003). The existing data suggest that the regulation of N metabolism is related to Cd adaptation, and it can be speculated that plants could better tolerate Cd when N supply is optimal.

Studies on dioecious plants are very interesting, as such species have important roles in terrestrial ecosystems. Already Darwin (1877) recognized that reproductive differentiation could result in secondary sexual dimorphism and sex-specific resource demands. Females are generally considered as having to make a higher reproductive effort than males, because they produce fruits in addition to flowers. Spatial segregation is common in dioecious species, with females occurring at higher frequencies in mesic or high nutrient habitats, and males predominating on xeric or low nutrient sites (Dawson and Ehleringer, 1993). The pattern of sexual segregation is beneficial for meeting sex-specific resource demands and reducing intraspecific competition (Eppley, 2006; Li *et al.*, 2007). Long-term adaptive evolution in distinct habitats and different reproduction costs between the sexes presumably result in physiological specialization and also in different stress sensitivity. During recent years, many studies have shown that females are usually sensitive to stressful environments (Xu *et al.*, 2008; Zhao *et al.*, 2009; Chen *et al.*, 2010; Zhang *et al.*, 2010, 2011). However, there is little information about sex-specific performance when dioecious species are exposed to environmental pollution, such as Cd, especially when combined with N deposition.

Populus species have been regarded as potentially promising candidates in phytoextraction and phytoremediation of Cd due to their fast growth, high biomass, extensive root mass, and low impact on food chains. In the present study, *P. yunnanensis* Dode., a native dioecious species in southwest China, which plays an important role in local aforestation and ecological restoration, is employed as a model species to investigate growth, physiological, biochemical, and ultrastructural responses to Cd stress, N deposition, and their combination. On the basis of the existing knowledge of sex-specific resource demands associated with reproduction in males and females, it is hypothesized that males have a higher tolerance to Cd than do females and that there are sexual differences in responses to Cd stress when combined with N deposition. The aims of the study were to answer the following questions. (i) Are females more sensitive to Cd stress and do they suffer more negative effects on, for example, gas exchange, than do males? (ii) Does N deposition mitigate Cd toxicity and cause sexual differences in responses to Cd?

Materials and methods

Plant materials and experimental design

Healthy cuttings of *P. yunnanensis* were collected from their natural habitats of Meigu (103°06′E, 28°18′N) in southwest Sichuan, China. Meigu is not only the major distribution region of *P. yunnanensis*, but is also rich in minerals, such as calamine, copper–zinc ore, and lead–zinc ore. Cd pollution is common in this region, especially in areas of mining and smelting, some of which were even contaminated with >50 mg Cd kg^{-1} dry soil. Mean altitude, mean annual rainfall, average temperature, maximum temperature, and minimum temperature in the region are 2300 m, 1115 mm, 10.1 °C, 17.3 °C, and 1.4 °C, respectively. A total of 30 male trees and 30 female trees were collected from 15 populations which cover the whole distribution region of *P. yunnanensis* in Meigu. The trees collected from all populations share similar conditions of water and soil nutrients. The cuttings were planted in March 2010. Female and male cuttings ~40 cm high were replanted into 10.0 l plastic pots filled with 12 kg of homogenized soil. The properties of the soil used in this study were as follows (based on kg^{-1} dry soil): pH 7.1, organic carbon 18.6 g, total N 1.75 g, hydrolysable N 132.05 mg, available phosphorus 2.68 g, total potassium 18.79 g, organic matter 23.85 g, and Cd content 0.08 mg. The cuttings were grown in a naturally lit 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 treatment period at the Chengdu Institute of Biology (CIB), the Chinese Academy of Sciences (CAS).

The experiment was a completely randomized design with eight factorial combinations of two levels of sex, Cd, and N deposition, respectively. A total of 100 healthy cuttings chosen from each sex were used for the study. These cuttings were from five populations selected from 15 populations randomly. A female tree and a male tree in each population were also chosen randomly. Each sex and treatment contained 25 cuttings [i.e. five replicates (five different populations), with five cuttings in each replicate (five cuttings from each population)]. Therefore, female and male cuttings were from the same wild populations in both controls and other treatments. In the Cd treatment, deionized water containing 100 μM $CdCl_2{\cdot}2.5H_2O$ was evenly added to the pots every day during the first 2 weeks of the treatment, and the final Cd level reached 50 mg $CdCl_2{\cdot}2.5H_2O$ kg^{-1} dry soil. In a parallel experiment, N deposition was supplied by adding an equal volume of aqueous solution with dissolved NH_4NO_3 similarly to in the Cd treatment every day during the first 2 weeks of the treatment. The concentration of the applied NH_4NO_3 was based on the N deposition level in natural habitats (i.e. 6 g N m^{-2} $year^{-1}$), the proportion of rainfall during the treatment period relative to the annual rainfall, and the area of soil in the pot. In the treatment to reveal the Cd and N deposition interaction, deionized water containing both $CdCl_2{\cdot}2.5H_2O$ and NH_4NO_3 was applied. At the same time, control individuals received equal quantities of deionized water. The treatments started on 15 May 2010, and the plants were harvested on 15 August 2010.

Growth measurements

At the end of the experiment, a cutting was selected randomly from five cuttings of each replicate, and thus there were five cuttings in total in each sex and treatment used for the measurement of biomass. The cuttings were harvested and separated into leaves, stems, and roots. Biomass samples were separately oven-dried (70 °C for 48 h) to constant weight and weighed. Dry matter accumulation (DMA) was then calculated.

Chlorophyll fluorescence and gas exchange measurements

A cutting was selected randomly from five cuttings of each replicate, and thus there were five cuttings in total in each sex and treatment used for the following measurements. The fourth fully expanded and exposed young leaf of each cutting, which was freshly formed after treatments, was used for chlorophyll fluorescence measurements. Chlorophyll fluorescence kinetics parameters [F_v/F_m, the variable and maximum fluorescence; and Yield, the effective quantum yield of photosystem II (PSII)] were measured and calculated according to van Kooten and Snel (1990) with a PAM chlorophyll fluorometer (PAM 2100, Walz, Effeltrich, Germany). First, leaf samples were placed in the dark for 30 min using an aluminium foil cover. The minimal fluorescence yield (F_0) and the maximal fluorescence yield (F_m) were measured. Then, the leaves were illuminated with actinic light at an intensity of 250 μmol m^{-2} s^{-1}, which was the light intensity inside the greenhouse at the time of the measurements. The actinic light was removed and the minimal fluorescence (F_0') was measured by illuminating the leaves with 3 s of far-red light. A saturating white light pulse of 8000 μmol m^{-2} s^{-1} was applied for 0.8 s when F_m and maximal fluorescence (F_m') were measured. Subsequently, the net photosynthesis rate (A), stomatal conductance (g_s), and transpiration rate (E) were measured with a portable photosynthesis measuring system (LI-6400; LI-COR Inc., Lincoln, NE, USA). The measurement conditions were as follows: leaf temperature, 25 °C; leaf to air vapour pressure deficit, 1.5±0.5 kPa; photosynthetic photon flux density (PPFD), 1500 μmol m^{-2} s^{-1}; relative air humidity, 50%; and ambient CO_2 concentration, 400±5 μmol mol^{-1}. Once the apparent steady-state gas exchange was achieved, the steady-state data were recorded.

Enzyme extractions and activity assays

The fourth fully expanded leaves randomly selected from each sex and treatment were used for the measurements of the following parameters, and also for the transmission electron microscopy (TEM) observations. Leaf samples (0.3 g of fresh leaves) were ground in liquid nitrogen and extracted with 50 mM potassium phosphate buffer (pH 7.8), containing 0.1 mM EDTA, 1% (w/v) polyvinylpyrrolidone (PVP), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.2% (v/v) Triton X-100, for the measurements of superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11), and glutathione reductase (GR; EC 1.6.4.2). For APX, 5 mM ascorbate was included in the extraction buffer. The extracts were centrifuged at 12 000 *g*, 4 °C for 15 min. The supernatants were used for the enzyme activity assays. All operations were performed at 0–4 °C. The soluble protein concentration was quantified as described by Bradford (1976), using bovine serum albumin as a standard.

The total SOD activity was determined by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture with a total volume of 3 ml contained 0.3 ml each of 20 μM riboflavin, 150 mM L-methionine, 600 μM NBT, and enzyme extract containing 100 μg of proteins. The reaction was started with the addition of riboflavin and carried out for 30 min under an irradiance of 170 μmol m^{-2} s^{-1} provided by a white fluorescent lamp. A system devoid of enzymes served as a negative control. SOD was measured when monitored at 560 nm using a spectrophotometer (Unicam UV-330, Unicam, Cambridge, UK). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT.

POD activity was assayed in 3 ml of 50 mM potassium phosphate buffer (pH 6.5) containing 40 mM guaiacol, 10 mM H_2O_2, and enzyme extract containing 100 μg of proteins at 25 °C. POD was measured when monitored at 436 nm for 3 min using a spectrophotometer. Activity was based on the rate of tetraguaiacol production using an extinction coefficient of 25.5 mM^{-1} cm^{-1}. One unit of POD was defined as the amount of enzyme that oxidizes 1 mmol of guaiacol min^{-1} per mg of protein.

APX was assayed in a total volume of 3 ml containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM sodium ascorbate, 2.5 mM H_2O_2, and enzyme extract containing 100 μg of proteins. APX was measured when monitored at 290 nm for 3 min using a spectrophotometer. Activity was based on the rate of oxidized ascorbate production using an extinction coefficient (ϵ=2.8 mM^{-1} cm^{-1}). One unit of APX was defined as the amount of enzymes that breaks down 1 μmol of ascorbate min^{-1} mg^{-1} of protein.

GR was assayed in a total volume of 3 ml of a mixture containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG), and enzyme extract containing 200 μg of proteins. GR was measured when monitored at 340 nm for 3 min using a spectrophotometer. GR activity was based on the the rate of decrease in NADPH using an extinction coefficient (ϵ=6.2 mM^{-1} cm^{-1}). One unit of GR was defined as the amount of enzymes that oxidize 1 nmol of NADPH min^{-1} mg^{-1} of protein.

Determination of reactive oxygen species and lipid peroxidation

The same leaf tissue samples as used for enzyme measurements were used for determination of $O_2\cdot^-$, H_2O_2, and thiobarbituric acid-reactive substances (TBARS). The measurements of superoxide radicals ($O_2\cdot^-$) followed the method of Lei *et al.* (2006). Samples reacted with 1 ml of hydroxylamine hydrochloride for 1 h, then 1 ml of *p*-aminobenzene sulphonic acid and 1 ml of α-naphthylamine were added, and the solution was kept at 25 °C for 20 min. The mixture was measured under 530 nm using $NaNO_2$ as the standard curve. The H_2O_2 content was determined as a H_2O_2–titanium complex resulting from the reaction of tissue H_2O_2 with titanium tetrachloride following the method of Brennan and Frekel (1977). The H_2O_2 concentration was measured when monitored at 410 nm using a spectrophotometer. Absorbance values were calibrated with a standard curve generated using known concentrations of H_2O_2.

Oxidative damage to lipids in leaves was expressed as equivalents of TBARS content following the description of Hodges *et al.* (1999). Leaves (~1 g) were homogenized in 10 ml of 10% trichloroacetic acid (TCA) and centrifuged at 12 000 *g* for 10 min. A 2 ml aliquot of 0.6% thiobarbituric acid (TBA) in 10% TCA was added to 2 ml of the supernatant. Test tubes filled with the mixture were heated in boiling water for 15 min, and quickly cooled in an ice bath. Then, the mixture was centrifuged at 12 000 *g* for 10 min. TBARS were determined after monitoring at 450, 532, and 600 nm using the spectrometer. The extraction solvent was used as the blank.

Leaf Cd determination

Leaf samples were washed thoroughly with deionized water, and then dried at 80 °C until a constant weight was reached. Dried samples were ground to fine powder and passed through a 100 mesh screen. Determination of leaf Cd was made by atomic absorption spectrophotometry (Analyst 300; Perkin Elmer, Germany) on nitric–perchloric acid (3:1, v/v) digests of five replicate samples from leaf tissue.

Determination of ascorbate and glutathione and their redox reactions

Ascorbate (ASC) and dehydroascorbate (DHA) were measured according to Kampfenkel *et al.* (1995) with minor modifications. Briefly, total ascorbate (TA) (ASC plus DHA) was determined after reduction of DHA to ASC with dithiothreitol (DTT), and the concentration of DHA was estimated from the difference between the TA pool and ASC. Leaf samples (0.3 g) were homogenized in 6% TCA pre-chilled on ice. The homogenate was then centrifuged at 12 000 *g* for 10 min and the resulting supernatant was used for the determination of TA and ASC. The reaction mixture for the TA pool contained a 0.1 ml aliquot of the supernatant, 0.25 ml of 50 mM phosphate buffer (pH 7.5) containing 2.5 mM EDTA, and 0.05 ml of 10 mM DTT. After incubation for 10 min at room temperature, 0.05 ml of 0.5% *N*-ethylmaleimide was added to remove excess DTT. ASC was determined in a similar reaction mixture except that 0.1 ml of H_2O was added rather than DTT and *N*-ethylmaleimide. Colour was developed in both reaction mixtures after the addition of the following reagents: 0.2 ml of 10% TCA, 0.2 ml of 44% *ortho*-phosphoric acid, 0.2 ml of 4% α, α'-dipyridyl in 70% ethanol, and 0.3% (w/v) $FeCl_3$. After vortexing, the mixture was incubated at 40 °C for 40 min. Then, TA and ASC were determined when monitored at 525 nm using a spectrophotometer. A standard curve was developed based on ASC in the range of 0–50 μg ml^{-1}.

Total glutathione (TG) [reduced glutathione (GSH) plus GSSG] and GSSG were determined by the 5, 5′-dithiobis-nitrobenzoic acid (DTNB)–GR recycling procedure (Loggini *et al.*, 1999). GSSG was reduced to GSH by the action of GR and NADPH, whereas GSH was oxidized by DTNB to give GSSG and 5-thio-2-nitrobenzene (TNB). A total of 0.3 g of leaf tissue was homogenized in ice-cold 5% sulphosalicylic acid. The homogenate was filtered through four layers of cheesecloth and centrifuged at 10 000 *g* for 10 min. The supernatant was used for the GSSG and TG assays. GSSG was determined from the sample after removal of GSH by 2-vinylpyridine derivatizations. The reaction mixture contained 0.5 M sodium phosphate buffer (pH 7.5), 2.5 mM EDTA, 0.25 mM NADPH, 6 mM DTNB, and 1 U of GR in a total volume of 1 ml. TG and GSSG were determined when monitored at 412 nm for 3 min using the spectrophotometer. A standard curve in the range of 0–100 μM GSSG was used. GSH was determined by subtraction of GSSG from the TG.

Determination of free amino acids and non-protein thiols

For the free amino acid (FAA) determination, 0.5 g of fresh leaves were ground with 3 ml of 3% sulphosalicylic acid and extracted in boiling water for 10 min. After cooling to room temperature, the extract was centrifuged at 5000 *g* at 4 °C for 10 min. Finally, the supernatants were collected and used for the FAA assays. The quantitative measurement of the total FAAs was conducted using the ninhydrin reaction (Correia *et al.*, 2005). Approximately 2 ml of buffered ninhydrin solution (0.8 g of ninhydrin and 0.12 g of hydrindantin dissolved in 30 ml of 2-methoxyethanol plus 10 ml of 4 M acetate buffer, pH 5.5) was added to 1 ml of supernatant and heated in boiling water for 15 min. The mixture was cooled to room temperature and 3 ml of 50% ethanol was added. After 10 min, FAAs were measured when monitored at 570 nm using a spectrophotometer. The amount of FAAs was determined by reference to a standard curve that was previously prepared with arginine.

The non-protein thiol (NP-SH) content was measured following the method of Ellman (1959). Leaf tissue (0.5 g) was homogenized in 6.67% 5'-sulphosalicylic acid. After centrifugation at 10 000 *g* for 10 min at 4 °C, the supernatant reacted with the Ellman reagent. The NP-SH content was measured when monitored at 412 nm using a spectrophotometer.

Transmission electron microscopy observations

Small leaf sections (1–2 mm in length) were fixed with 3% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2) for 6–8 h under 4 °C, post-fixed in 1% osmium tetroxide for 1 h, and immersed in 0.1 M phosphate buffer (pH 7.2) for 1–2 h. The leaflets were then dehydrated in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100%) and embedded in epon–araldite. Ultra-thin sections (80 nm) were sliced, stained with uranyl acetate and lead citrate, and mounted on copper grids for viewing in the H-600IV TEM (Hitachi, Tokyo, Japan).

Statistical analyses

All data were analysed with the software Statistical Package for the Social Sciences (SPSS) version 16.0. Three-way analyses of variance (ANOVAs) were employed to test the overall effects of sex, Cd, and N deposition on growth, physiological, and biochemical parameters. All data were checked for normality and the homogeneity of variances, and log-transformed to correct deviations from these assumptions when needed. Post-hoc comparisons were tested using the Tukey's test at a significance level of $\alpha=0.05$.

Results

Sexual differences in morphology

In the present study, females and males of *P. yunnanensis* were exposed to Cd in a soil culture and in a controlled environment for 3 months to minimize experimental variation and clearly distinguished Cd exposure responses. High Cd concentration in the soil solution did not induce rapid visible injuries in either sex until chlorosis in the centre of the apex of young leaves in females under Cd stress alone was first observed after 2 months. However, the DMA of plants was affected to some extent at the end of treatments. The DMA significantly decreased in females, whereas it was hardly affected in males under Cd stress when compared with the controls. In contrast, DMA significantly increased in males, whereas it was hardly affected in females under N deposition. DMA of both sexes did not vary significantly under combined treatment when compared with the controls. Males showed significantly higher DMA than did females under N deposition, while there were no significant differences in DMA between the sexes under control conditions. Additionally, DMA as a variable was not only affected significantly by the single factors, but was also affected significantly by the interactive effect of Cd×N deposition and sex×Cd×N deposition based on ANOVA (Table 1).

Sexual differences in chlorophyll fluorescence and gas exchange

Cd stress alone and the combined treatment significantly decreased F_v/F_m and Yield in both sexes when compared with the controls, especially in females. Significant sexual differences in F_v/F_m and Yield existed only under Cd stress, with males displaying higher F_v/F_m and Yield than did females, whereas there were no significant differences in either F_v/F_m or Yield between the sexes under control conditions. Based on ANOVA, both F_v/F_m and Yield were

Table 1. Dry matter accumulation (DMA), maximum efficiency of PSII (F_v/F_m), the effective quantum yield of PSII (Yield), net photosynthesis rate (A), stomatal conductance (g_s), and transpiration rate (E) in *P. yunnanensis* females and males, as affected by Cd, N deposition, and their combination, F_s, sex effect; F_m, Cd effect; F_n, N deposition effect; $F_s \times F_m$, the interactive effect of sex and Cd; $F_s \times F_n$, the interactive effect of sex and N deposition; $F_m \times F_n$, the interactive effect of Cd and N deposition; $F_s \times F_m \times F_n$, the interactive effect of sex, Cd, and N deposition.

Each value is the mean ±SE (n=5). Values followed by the same letter in the same column are not significantly different according to Tukey's test. NS, not significant; *P <0.05; **0.01 ≤P <0.001; and ***P ≤ 0.001.

N deposition (g N m^{-2} $year^{-1}$)	$CdCl_2 \cdot 2.5H_2O$ (mg kg^{-1} dry soil)	Sex	DMA (g)	F_v/F_m	Yield	A (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	E (mmol m^{-2} s^{-1})
0	0	Female	31.91±1.20 bc	0.80±0.004 a	0.71±0.004 a	15.43±0.24 ab	0.41±0.01 cd	5.44±0.24 bcd
0	0	Male	30.74±0.88 c	0.81±0.003 a	0.73±0.003 a	15.63±0.26 ab	0.49±0.02 c	6.53±0.40 ab
0	50	Female	21.31±1.53 d	0.67±0.010 d	0.57±0.018 c	9.53±0.36 d	0.23±0.02 f	3.81±0.21 e
0	50	Male	26.29±0.97 cd	0.74±0.007 b	0.65±0.007 b	12.20±0.61 c	0.31±0.01 e	4.77±0.11 de
6	0	Female	37.46±0.95 b	0.79±0.004 a	0.72±0.003 a	16.43±0.73 a	0.58±0.02 b	6.07±0.30 abcd
6	0	Male	44.32±1.51 a	0.79±0.005 a	0.70±0.006 a	17.13±0.58 a	0.68±0.02 a	6.87±0.34 a
6	50	Female	28.55±0.74 c	0.69±0.009 cd	0.63±0.006 b	13.03±0.86 bc	0.36±0.02 de	5.20±0.17 cd
6	50	Male	30.36±1.45 c	0.72±0.004 bc	0.65±0.008 b	14.83±0.37 abc	0.35±0.01 de	6.26±0.26 abc
		P: F_s	**	***	**	**	***	***
		P: F_m	***	***	***	***	***	***
		P: F_n	***	*	NS	***	***	***
		P: $F_s \times F_m$	NS	***	***	*	*	NS
		P: $F_s \times F_n$	NS	*	***	NS	NS	NS
		P: $F_m \times F_n$	*	*	**	*	**	*
		P: $F_s \times F_m \times F_n$	**	NS	NS	NS	*	NS

significantly affected by sex, Cd, and the interactive effect of sex×Cd, sex×N deposition, and Cd×N deposition. F_v/F_m was also significantly affected by N deposition (Table 1). On the other hand, Cd stress alone significantly decreased A, g_s, and E in both sexes when compared with the controls, especially in females. However, these parameters increased to some degree under N deposition. The combined treatment hardly affected A, g_s, and E in either sex when compared with the controls, except for g_s of males. Males showed significantly higher A and g_s than did females under Cd stress alone, whereas there were no significant differences in either A or g_s between the sexes under control conditions. Based on ANOVA, A, g_s, and E were affected by sex, Cd, N deposition, and the interaction Cd×N deposition. In addition, A was significantly affected by the interaction sex×Cd, while g_s was significantly affected by the interactive effect of sex×Cd and sex×Cd×N deposition (Table 1).

Sexual differences in antioxidant enzymes

The activities of SOD significantly decreased in females under Cd stress and in males under N deposition when compared with the controls (Fig. 1a). Males showed significantly higher SOD activity than did females under control conditions, Cd stress alone, and under the combined treatment (Fig. 1a). The sexual difference in SOD activity under Cd stresss alone was greater than that under control conditions. In respect to POD, there was a 52.7% increase in females in response to the combined treatment, while POD was hardly affected by Cd stress alone and N deposition compared with control females. In contrast, POD of males increased 257.2% and 258.0% under Cd stress alone and under the combined treatment, respectively, compared with control males. Females exhibited higher POD than did males under both control and N deposition conditions (Fig. 1b). Based on ANOVA, SOD and POD were significantly affected by sex, Cd, N deposition, and the interactive effect of sex×Cd and sex×N deposition. POD was also significantly affected by the interaction sex×Cd×N deposition (Table 2).

APX activities of females significantly increased under the combined treatment compared with control females. In males, both Cd stress alone and the combined treatment induced a significant increase in APX compared with control males (Fig. 1c). There were no significant sexual differences in APX under control conditions and other treatments. As regards GR, Cd stress alone induced a significant decrease in females compared with control females (Fig. 1d). However, all treatments hardly affected the GR activity of males. Males displayed significantly higher GR than did females under Cd stress alone, N deposition, and the combined treatment, while there were no significant differences in GR between the sexes under control conditions. Moreover, based on ANOVA, APX was significantly affected by Cd, N deposition, and the interaction sex×Cd×N deposition. GR was significantly affected by sex, Cd, N deposition, and the interaction Cd×N deposition (Table 2).

Sexual differences in reactive oxygen species and lipid peroxidation

Compared with the controls, Cd stress alone and the combined treatment induced a higher level of $O_2^{\cdot -}$, H_2O_2, and TBARS in both sexes, especially in females (Fig. 2a–c).

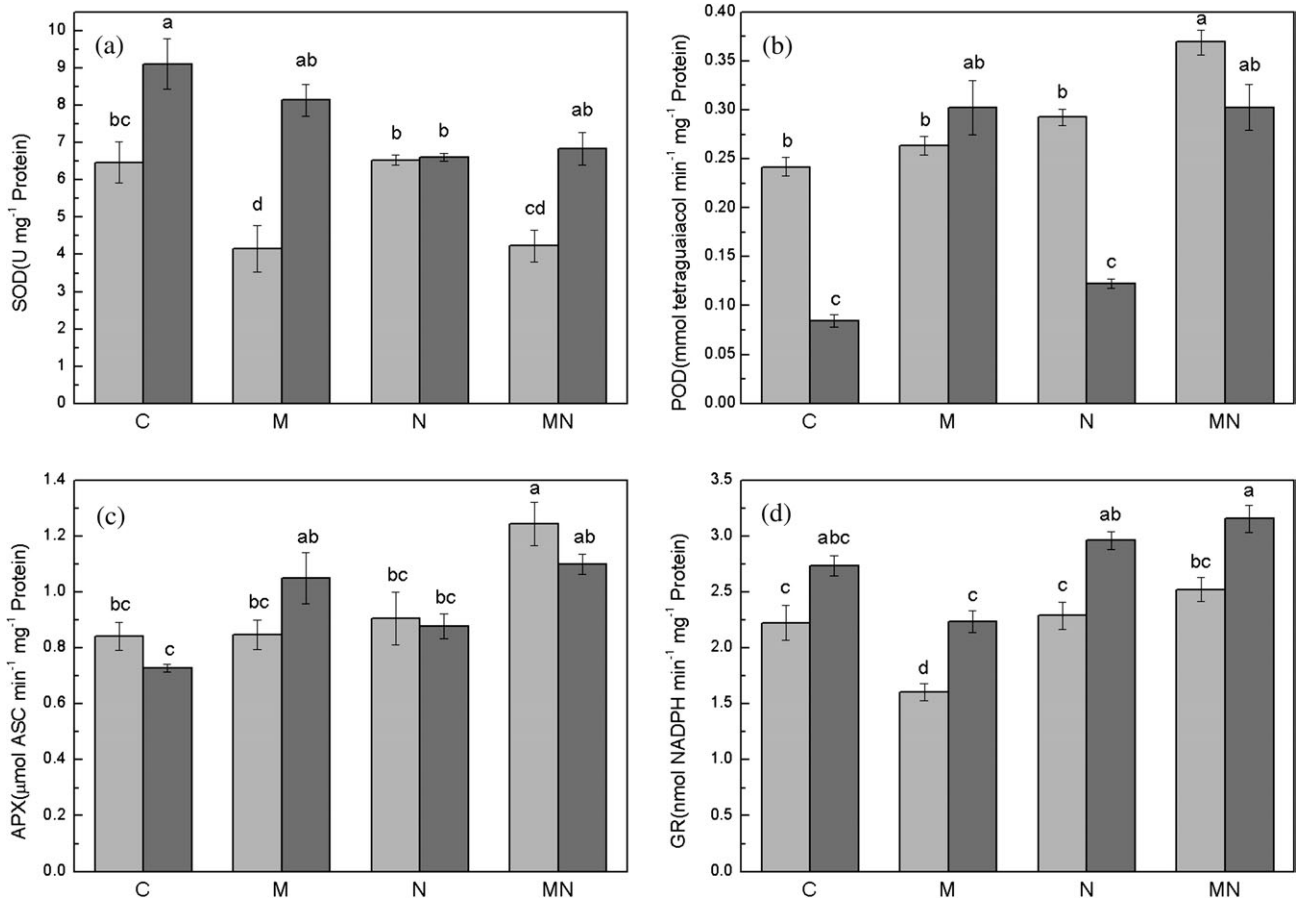

Fig. 1. Activities of superoxide dismutase (SOD) (a), peroxidase (POD) (b), ascorbate peroxidase (APX) (c), and glutathione reductase (GR) (d) in females (light grey) and males (dark grey) of *P. yunnanensis* in the control condition (C), and as affected by Cd stress alone (M), N deposition (N), and the combined treatment (MN). Each value is the mean ±SE (n=5). The values not sharing the same letters are significantly different if P <0.05 according to Tukey's test.

Table 2. Statistical significance of single and interactive effects of sex, Cd, and N deposition on physiological and biochemical parameters based on three-way ANOVA, F_s, sex effect; F_m, Cd effect; F_n, N deposition effect; $F_s \times F_m$, the interactive effect of sex and Cd; $F_s \times F_n$, the interactive effect of sex and N deposition; $F_m \times F_n$, the interactive effect of Cd and N deposition; $F_s \times F_m \times F_n$, the interactive effect of sex, Cd, and N deposition.

NS, not significant; *P <0.05; **0.01 ⩽P <0.001; and ***P ⩽ 0.001.

Parameter	P: F_s	P: F_m	P: F_n	P: $F_s \times F_m$	P: $F_s \times F_n$	P: $F_m \times F_n$	P: $F_s \times F_m \times F_n$
SOD	***	***	*	**	**	NS	NS
POD	***	***	***	***	*	NS	*
APX	NS	***	**	NS	NS	NS	*
GR	***	*	***	NS	NS	***	NS
$O_2 \cdot^-$	***	***	NS	NS	NS	NS	NS
H_2O_2	***	***	*	***	*	**	NS
TBARS	***	***	***	**	NS	**	NS
Cd	**	***	***	NS	NS	***	NS

Under Cd stress alone, $O_2 \cdot^-$, H_2O_2, and TBARS increased 43.9, 72.9, and 78.4%, respectively, in females but only 23.2, 36.0, and 41.4%, respectively, in males. Under the combined treatment, $O_2 \cdot^-$, H_2O_2, and TBARS increased ~26.2, 34.7, and 26.9%, respectively, in females but only 17.1, 23.1, and 12.0%, respectively, in males. Females showed significantly higher $O_2 \cdot^-$, H_2O_2, and TBARS than did males under Cd stress alone. Under the combined treatment, females showed significantly higher H_2O_2 and TBARS than did males. In contrast, there were no significant differences in either $O_2 \cdot^-$ or TBARS between the sexes under control conditions. Although females showed significantly higher H_2O_2 than males under control conditions, the sexual differences in H_2O_2 under Cd stresss alone and the combined treatment were greater than those under control conditions. However, N deposition did not induce significant changes in these parameters. Moreover, based on ANOVA, $O_2 \cdot^-$ was significantly affected by sex and Cd. Both H_2O_2 and TBARS were significantly affected by sex, Cd, N deposition, and the interactive effect of sex×Cd and Cd×N deposition. Also H_2O_2 was significantly affected by the interaction sex×N deposition (Table 2).

Sexual difference in leaf Cd content

Cd stress alone and the combined treatment induced a significant increase in leaf Cd in both sexes when compared with the controls. There was a significant difference between

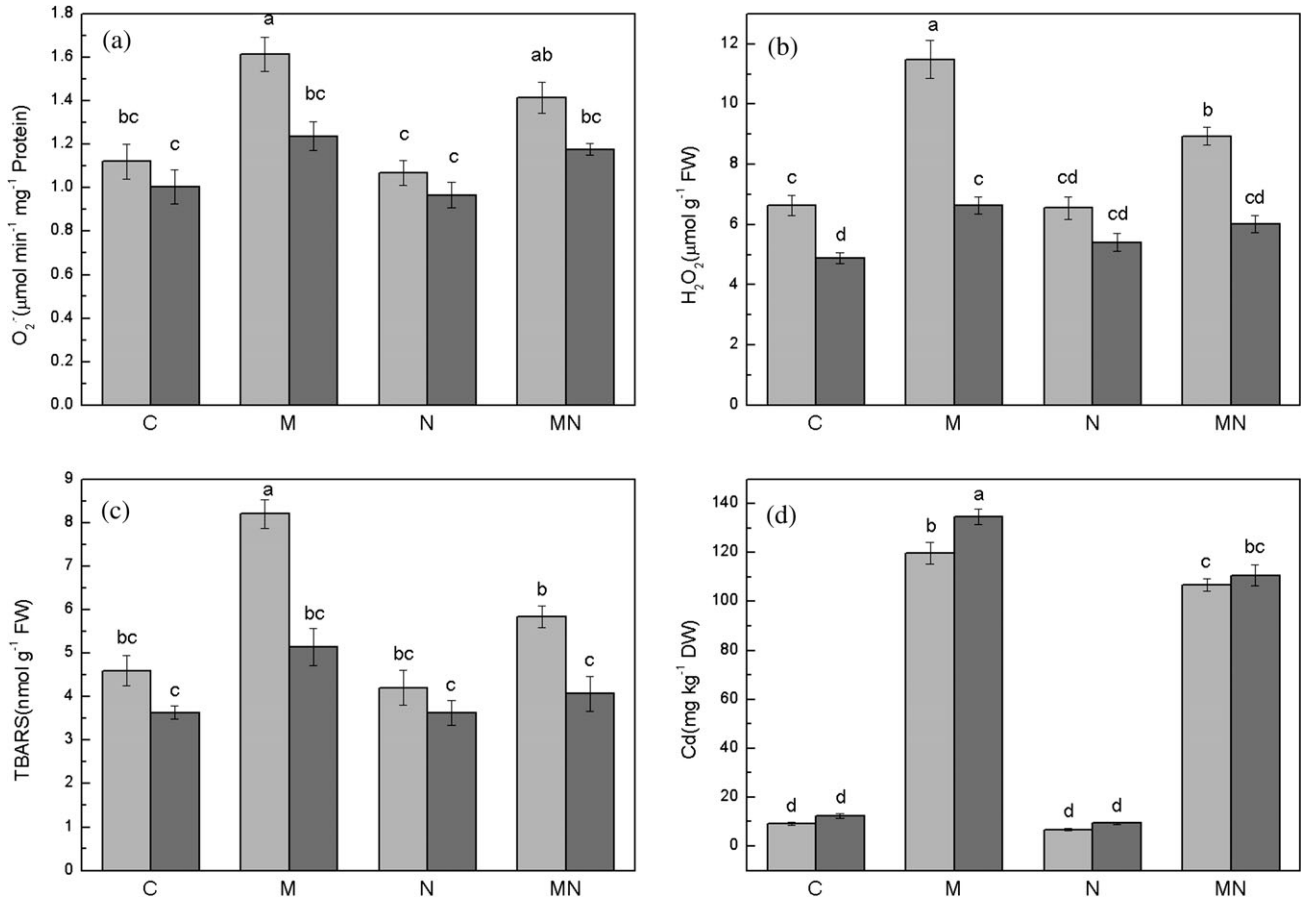

Fig. 2. The production ratio of superoxide radicals ($O_2\cdot^-$) (a), hydrogen peroxide content (H_2O_2) (b), content of thiobarbituric acid-reactive substance (TBARS) (c), and leaf Cd content (d) in females (light grey) and males (dark grey) of *P. yunnanensis* in the control condition (C), and as affected by Cd stress alone (M), N deposition (N), and the combined treatment (MN). Each value is the mean ±SE (n=5). The values not sharing the same letters are significantly different if P <0.05 according to Tukey's test.

the sexes in leaf Cd under Cd stress alone, males showing a significantly higher content of leaf Cd than females, while there was no significant difference in leaf Cd between the sexes under control conditions (Fig. 2d). Based on ANOVA, leaf Cd was significantly affected by sex, Cd, N deposition, and the interaction Cd×N deposition (Table 2).

Sexual differences in ascorbate and glutathione contents and redox reactions

Cd stress significantly decreased TA and ASC/DHA in both sexes when compared with the controls. Under Cd stress alone, TA of females and males decreased ~29.9% and 11.2%, respectively, while ASC/DHA of females and males decreased ~59.9% and 47.7%, respectively. The combined treatment induced 40.0% and 14.9% decreases in ASC/DHA of females and males, respectively. Males showed significantly higher TA under Cd stress alone than females, while there were no significant differences in TA between the sexes under control conditions. Although females exhibited significantly higher ASC/DHA than males under control conditions, there were no significant sexual differences in ASC/DHA under Cd stress alone and the combined treatment (Table 3). Based on ANOVA, TA and ASC/DHA were significantly affected by sex, Cd, N deposition, and the interaction of Cd×N deposition. ASC/DHA was also significantly affected by the interaction sex×Cd (Table 3).

Both Cd stress alone and the combined treatment significantly increased TG but decreased GSH/GSSG in both sexes when compared with the controls. Cd stress alone and the combined treatment induced 33.4% and 37.4% increases in TG, respectively, of females, while 46.4% and 42.9% increases, respectively, were detected in males (Table 3). In contrast, Cd stress alone and the combined treatment induced 34.0% and 23.5% decreases in GSH/GSSG, respectively, of females but only 21.8% and 8.9% decreases, respectively, in males. Males showed significantly higher TG than females under Cd stress alone, while there were no significant differences in TG between the sexes under control conditions. In contrast, females displayed significantly higher GSH/GSSG than males under control conditions, while there were no significant sexual differences in GSH/GSSG under Cd stress alone and the combined treatment. Based on ANOVA, both parameters were significantly affected by sex, Cd, N deposition, and the interactive effect of sex×Cd and Cd×N deposition (Table 3).

Table 3. Total content of ascorbate (ASC+DHA) (TA), ratio of ASC to DHA (ASC/DHA), total content of glutathione (GSH+GSSG) (TG), ratio of GSH to GSSG (GSH/GSSG), free amino acids (FAAs), and content of non-protein thiols (NP-SHs) in *P. yunnanensis* females and males, as affected by Cd, N deposition, and their combination, F_s, sex effect; F_m, Cd effect; F_n, N deposition effect; $F_s \times F_m$, the interactive effect of sex and Cd; $F_s \times F_n$, the interactive effect of sex and N deposition; $F_m \times F_n$, the interactive effect of Cd and N deposition; $F_s \times F_m \times F_n$, the interactive effect of sex, Cd, and N deposition.

Each value is the mean ±SE (n=5). Values followed by the same letter in the same column are not significantly different according to Tukey's test. NS, not significant; *P <0.05; **0.01 ⩽P <0.001; and ***P ⩽ 0.001.

N deposition (g N m^{-2} $year^{-1}$)	$CdCl_2 \cdot 2.5H_2O$ (mg kg^{-1} dry soil)	Sex	TA (μmol g^{-1} FW)	ASC/DHA	TG (nmol g^{-1} FW)	GSH/GSSG	FAAs (mg g^{-1} FW)	NP-SHs (μmol g^{-1} FW)
0	0	Female	11.53±0.28 c	7.30±0.33 a	247.02±11.43 d	1.62±0.06 a	0.27±0.02 d	0.35±0.02 d
0	0	Male	13.20±0.47 abc	4.30±0.21 c	268.48±12.89 d	1.24±0.05 b	0.41±0.02 d	0.42±0.02 cd
0	50	Female	8.09±0.12 d	2.93±0.11 de	329.53±10.04 bc	1.07±0.05 bc	0.39±0.03 d	0.62±0.03 b
0	50	Male	11.73±0.50 c	2.25±0.16 e	392.95±13.53 a	0.97±0.04 c	0.58±0.02 c	0.80±0.06 a
6	0	Female	12.66±0.48 abc	5.94±0.14 b	298.18±8.73 cd	1.55±0.05 a	0.70±0.03 c	0.43±0.02 cd
6	0	Male	14.35±0.21 a	4.08±0.23 c	285.34±9.42 cd	1.26±0.04 b	1.26±0.03 a	0.41±0.02 cd
6	50	Female	11.88±0.49 bc	4.38±0.28 c	339.43±11.55 abc	1.24±0.06 b	0.86±0.02 b	0.54±0.04 bc
6	50	Male	13.77±0.51 ab	3.66±0.11 cd	383.76±10.36 ab	1.13±0.03 bc	0.96±0.04 b	0.69±0.04 ab
		P: F_s	***	***	**	***	***	***
		P: F_m	***	***	***	***	NS	***
		P: F_n	***	*	*	*	***	NS
		P: $F_s \times F_m$	NS	***	**	**	***	**
		P: $F_s \times F_n$	NS	NS	NS	NS	***	NS
		P: $F_m \times F_n$	**	***	*	*	***	**
		P: $F_s \times F_m \times F_n$	NS	NS	NS	NS	***	NS

Sexual differences in FAAs and NP-SHs

Compared with the controls, FAAs significantly increased in both sexes, except for females under Cd stress alone, under all treatments, especially under N deposition and the combined treatment. A significant sexual difference in FAAs was detected under Cd stress and under N deposition, and males exhibited higher FAA levels than did females under these treatments, while there were no significant differences in this parameter between the sexes under control conditions. Based on ANOVA, FAAs were significantly affected by sex, N deposition, and the interactive effect of sex×Cd, sex×N deposition, Cd×N deposition, and sex×Cd×N deposition (Table 3). On the other hand, NP-SHs significantly increased under Cd stress and under the combined treatment when compared with the controls, 79.4% and 53.8%, respectively, in females, but 92.2% and 66.1%, respectively, in males. Males exhibited a significantly higher level of NP-SHs under Cd stress alone, while there were no significant differences in NP-SHs between the sexes under control conditions. Based on ANOVA, NP-SHs were significantly affected by sex, Cd, and the interactive effect of sex×Cd and Cd×N deposition (Table 3).

Sexual differences detected in TEM observations

Typical elliptical chloroplasts with 8–15 grana in females and 6–13 grana in males were seen under control conditions by ultrastructural observations (Figs 3a, 4a). Each granum of both sexes was well developed and highly stacked with 15–30 thylakoids. In both sexes, there were starch granules in some chloroplasts. In contrast, under other conditions, chloroplast ultrastructure showed visible changes. Under Cd stress alone, the number of grana decreased to 2–6 with 6–12 thylakoids in each granum of females (Fig. 3b). In males, Cd stress alone resulted in a non-compact lamella structure, but there was no significant decrease in the number of either grana or thylakoids compared with control males (Fig. 4b). At the same time, 3–5 plastoglobuli emerged in both sexes under Cd stress alone. N deposition did not lead to a significant difference in the number of grana in either sex compared with the controls, but the number of thylakoids in each granum increased to 20–40 in females and 18–35 in males, respectively (Figs 3c, 4c). There were almost no starch granules in the chloroplasts of either sex under N deposition. When the plants were exposed to the combined treatment, 6–13 grana with 3–10 thylakoids in each granum were observed in females (Fig. 3d), whereas no significant decreases in the number of either grana or thylakoids were detected in males compared with control males (Fig. 4d).

Discussion

Sexual dimorphism in growth, chlorophyll fluorescence, and gas exchange

In the present study, both sexes of *P. yunnanensis* responded to Cd with retardation of plant growth, depression of the gas exchange rate, and impairments in the photosynthetic apparatus. On the one hand, stomatal closure and decreases in transpiration and photosynthesis rates have been well documented when plants are exposed to Cd (Pietrini *et al.*,

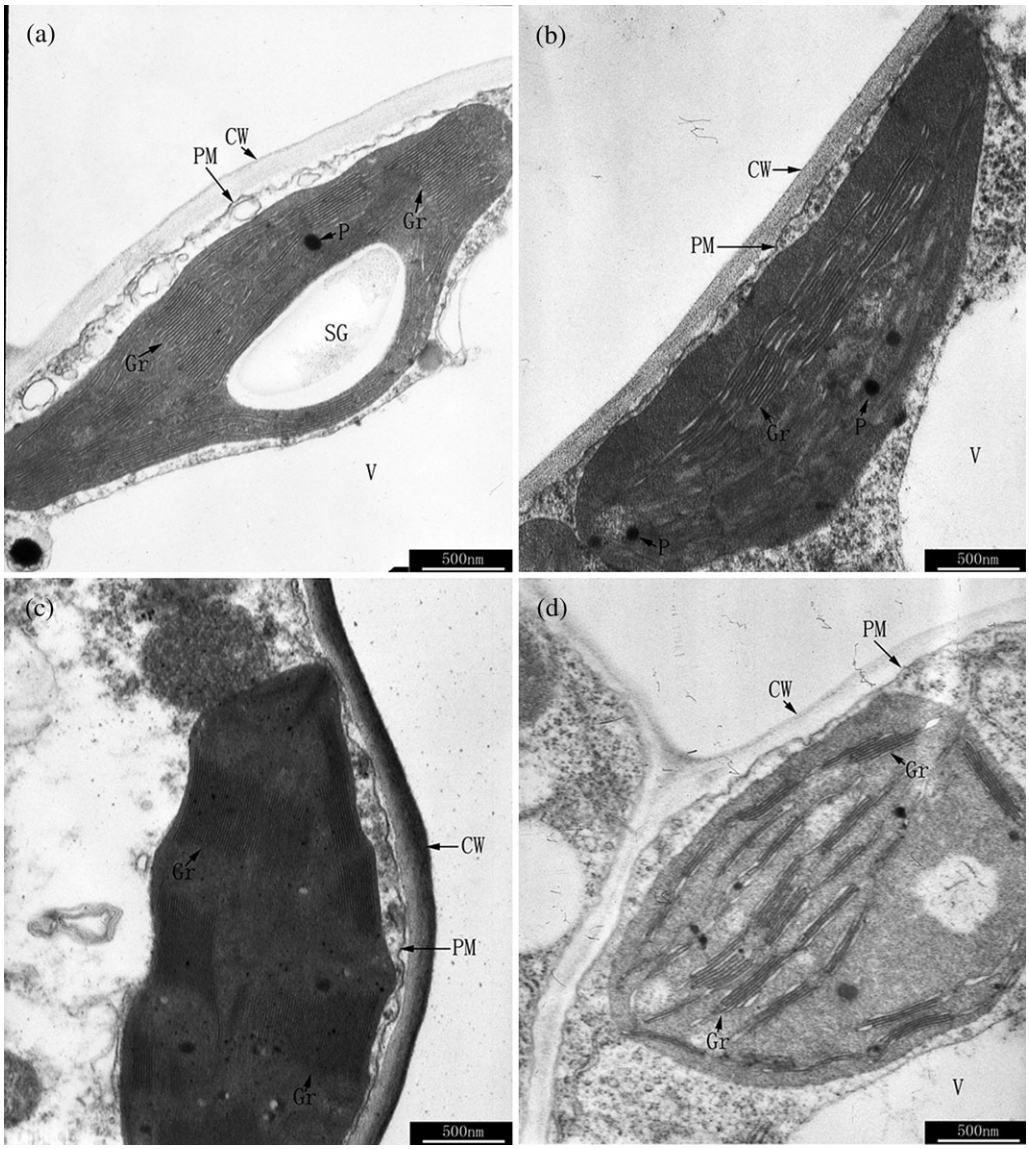

Fig. 3. Chloroplast ultrastructure in females of *P. yunnanensis* exposed to the control condition (a), Cd stress alone (b), N deposition (c), and the combined treatment (d). CW, cell wall; Gr, granum; M, mitochondrion; N, nucleus; NM, nuclear membrane; P, plastoglobulus; PM, plasma membrane; SG, starch granule; V, vacuole.

2003, 2010; Durand *et al.*, 2010). On the other hand, chlorophyll fluorescence, as an indicator of the photochemical efficiency of PSII, can provide insights into both the ability of plants to tolerate environmental stresses and the extent to which their photosynthetic apparatus has been damaged (Maxwell and Johnson, 2000). PSII has been proved to be sensitive to Cd, as shown by a decreased variable fluorescence (Küpper *et al.*, 2007; Solti *et al.*, 2008), which resulted from binding of Cd to several sites in PSII, reducing ferredoxin-$NADP^+$ oxidoreductase activity and arresting plastoquinone synthesis (Krupa and Baszynski, 1995; Sigfridsson *et al.*, 2004). In the present study, the noticeable decreases in F_v/F_m and Yield indicated that Cd disturbed the electron transport flow and reflected a disorder in PSII reaction centres of *P. yunnanensis*. Therefore, it can be concluded that inhibition of carbon assimilation resulted from stomatal limitation, PSII impairments, and disorganization of the chloroplast lamellar structure, observed using TEM. In addition, females showed larger decreases in DMA, F_v/F_m, Yield, A, and g_s than males under Cd stress alone, whereas no significant sexual differences in these traits were detected under control conditions. Thus, the results indicated that females suffered more negative effects induced by Cd than did males, which is in accordance with earlier studies showing that females are more sensitive than males under various stressful environments (Xu *et al.*, 2008; Chen *et al.*, 2010; Zhang *et al.*, 2011).

Many previous studies on the interactive effects of N availability and stress conditions have revealed conflicting results, for example a higher N status can lead to increased sensitivity to stress (Yao and Liu, 2007) or to a decreased sensitivity to stress conditions (Saneoka *et al.*, 2004). It can be concluded that the N effect on stresses depended mainly on the properties of the stresses, the exposure duration, the

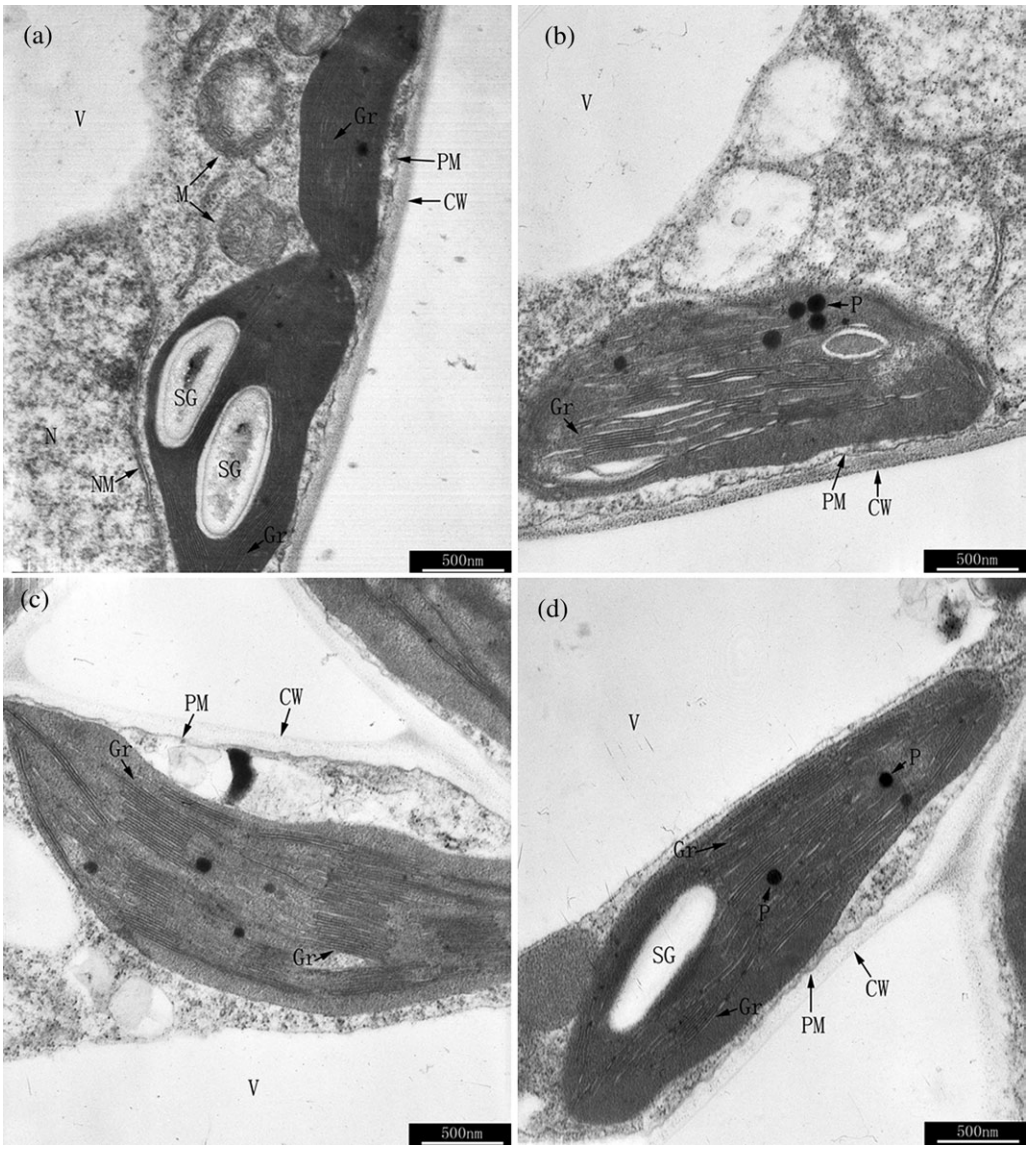

Fig. 4. Chloroplast ultrastructure in males of *P. yunnanensis* exposed to the control condition (a), Cd stress alone (b), N deposition (c), and the combined treatment (d). CW, cell wall; Gr, granum; M, mitochondrion; N, nucleus; NM, nuclear membrane; P, plastoglobulus; PM, plasma membrane; SG, starch granule; V, vacuole.

soil status, and the nutrient requirements of species. In the present study, both sexes showed higher gas exchange rates and more integrated structures of thylakoids, as reflected by TEM observations, under the combined treatment compared with Cd stress alone. These results suggested that N deposition had a mitigation effect on the Cd toxicity. In addition, it should be pointed out that sexual differences in F_v/F_m, Yield, A, and g_s decreased under the combined treatment compared with Cd stress alone. Therefore, the results suggest that Cd toxicity is N dependent, and sex-related differences in response to Cd would vary according to differences in soil N status.

Sexual dimorphism in antioxidant enzyme activities and ROS scavenging capabilities

Many previous studies have indicated that Cd, although it does not participate in Fenton-type ROS-producing reactions, can indirectly activate NADPH oxidases in membranes, disrupt the electron transport chain, and inhibit antioxidative enzymes, giving rise to oxidative bursts in plants (Olmos *et al.*, 2003; Garnier *et al.*, 2006). Specific enzymes, such as SOD, POD, APX, and GR, and non-enzyme low molecular weight molecules, such as ascorbate and glutathione, are two types of antioxidative systems to scavenge ROS and protect cells from oxidative stress. In the present study, SOD activities of both sexes were inhibited by Cd stress. Inhibition of SOD activities probably arises not only from enzyme oxidation induced by H_2O_2 (Sandalio *et al.*, 2001) but also from the inactivation effect, because Cd can competitively combine on cofactor sites, such as Cu/Zn SOD (Kieffer *et al.*, 2009). The results also showed that this inhibition effect was greater in females than in males when compared with the controls, which was the main reason for higher $O_2^{\cdot-}$ accumulation in females. In addition,

previous studies indicated that SOD isozymes (i.e. Cu/Zn-SOD, Mn-SOD, and Fe-SOD), which localized in different cell compartments, responded to Cd in transcription and expression differentially, and exhibited different sensitivity to oxidation modification (Romero-Puertas *et al.*, 2002, 2007; Rodríguez-Serrano *et al.*, 2009). Therefore, a further study, including analysis of the content and activity of specific isozymes, is required to clarify Cd toxicity and develop a deep understanding of different sexual antioxidative enzyme responses to Cd.

Ascorbate–glutathione cycle enzymes, such as APX and GR, play an important role in scavenging H_2O_2 through a series of coupled redox reactions using ascorbate and glutathione as substrates (Noctor and Foyer, 1998), whereas H_2O_2, as a signalling molecule, may induce the expression of APX (Schützendübel *et al.*, 2001). The present results showed that Cd induced an increase in the APX activity along with a rise in H_2O_2, but decreased GR in both sexes. The decrease in the GR activity was probably due to inactivation, because Cd has affinity for the sulphydryl groups of GR (Van Assche and Clijsters, 1990). Under Cd stress alone, females had a greater inhibition of the ascorbate–glutathione cycle due to lower GR and lower POD response compared with the controls, which inevitably affected detoxification of H_2O_2 and resulted in its higher accumulation in females than in males. In contrast, males with higher enzyme activities showed lower ROS levels and then lower lipid peroxidation, measured with TBARS, than did females. These findings suggest that Cd alters the equilibrium between ROS production and enzymatic defence reactions, resulting in greater oxidative stress in females than in males. The above-mentioned findings are in agreement with the conclusion of earlier studies that males can maintain higher enzyme activities to scavenge ROS under adverse environments, such as osmotic stress and chilling in *Populus* (Chen *et al.*, 2010; Zhang *et al.*, 2011).

Cellular redox imbalance is easily induced by Cd (Sharma and Dietz, 2009), which could disturb ROS generation/detoxication and induce the accumulation of ROS, leading to oxidative stress. The present results indicated that Cd decreased the ascorbate pool but increased the glutathione content, whereas the redox balance of both molecules was severely disturbed, since the ratio of the reduced state to the oxidized state sharply decreased under Cd stress. A decrease in ASC may be traced to a reduction in its synthesis. Kieffer *et al.* (2008) indicated that the expression of GDP-mannose-3', 5'-epimerase, a key enzyme in *de novo* ascorbate synthesis, decreases in response to Cd. The decreased activity of GR may be partially responsible for the decrease of GSH/GSSG and ASC/DHA under Cd stress. Although there were no significant differences in ASC/DHA and GSH/GSSG under Cd stress alone, females maintained significantly higher ASC/DHA and GSH/GSSG than males under control conditions. These results suggest that the redox balance of females is more sensitive to Cd stress than that of males. Combined with a greater decrease in ascorbate, the higher degree of imbalance in females than in males probably further accelerates the generation and accumulation of ROS in females. Under the combined treatment, the inhibition of SOD due to Cd was not significant in either sex, and GR activities of both sexes were greater than those under Cd stress alone. Moreover, N deposition rescued the cellular redox imbalance induced by Cd to some extent, shown as increases in ASC/DHA and GSH/GSSG when compared with Cd stress responses. Even so, injuries of females, evidenced by TBARS production and TEM observations, were higher than those of males under the combined treatment. Taken together, it can be concluded that the antioxidative system and cellular redox balance are more susceptible to disruption in females than in males under Cd stress. The antioxidative responses to Cd are N status dependent, and N availability could affect sexual differences in antioxidative enzymes and redox balance in *P. yunnanensis*.

Sexual dimorphism in Cd detoxification

Leaf Cd content of both sexes significantly increased under exposure to Cd stress irrespective of soil N status. Sexual dimorphism in leaf Cd content existed only under Cd stress alone, and males showed a higher leaf Cd content than did females. It was interesting to discover that N deposition increased *E*, but leaf Cd content decreased under the combined treatment, particularly in males. These results support the idea that Cd uptake may be dependent not only on transpiration flow (Perfus-Barbeoch *et al.*, 2002), and suggest that soil N status probably affects Cd uptake and translocation. In addition, growth improvement due to N deposition may dilute the leaf Cd content in both sexes.

Cd binding to sulphydryl groups of PCs is a fundamental mechanism of Cd detoxification. PCs are synthesized from GSH, and their amount can be estimated from the difference between NP-SHs and GSH. Many previous studies have indicated that Cd induces an increase in NP-SHs (Metwally *et al.*, 2003; Wawrzyński *et al.*, 2006), but the change in GSH in response to Cd is not coincidental (Metwally *et al.*, 2005). In the present study, NP-SHs and GSH increased in both sexes, while the increment in NP-SHs was more significant than the increase in GSH in response to Cd, which indicated that more PCs were synthesized for detoxification under exposure to Cd. The results also showed that the increment in PCs is greater in males than in females under Cd stress. Combined with a higher glutathione level, males showed a higher capability of detoxification despite males accumulating a higher leaf Cd content than females under such conditions. On the other hand, many previous studies have reported that FAAs play an important role in N recycling and reserve, and detoxification of Cd (Chaffei *et al.*, 2004; Sharma and Dietz, 2006). In the present study, Cd stress alone induced a slight increase in FAAs in females but a significant increase in males, while there was no significant sexual difference in FAAs under control conditions. Under such conditions, increased FAAs could be beneficial for osmoregulation and detoxification in males. Therefore, higher NP-SHs and FAAs played important roles in Cd detoxification and tolerance in both sexes, particularly in males.

In the present study, N deposition induced significantly higher FAA levels in both sexes under both N deposition alone and combined treatment. Higher FAA levels probably offered a better ability to chelate free Cd and scavenge ROS under the combined treatment. For example, free proline, which exhibited similar changes to FAAs in our treatments (data not shown), as one of the most important FAAs, has been suggested to function beneficially as an osmoregulator, metal chelator, and antioxidant in plant heavy metal stress tolerance (Mehta and Gaur, 1999; Siripornadulsil *et al.*, 2002). On the other hand, lower leaf Cd concentration under combined treatment induced toxicity of Cd to a lesser degree compared with Cd stress alone. It is believed that these are two important mechanisms for better performance of *P. yunnanensis* under combined treatment. In addition, it had been shown that N metabolism was sensitive to Cd (Wang *et al.*, 2008; Li *et al.*, 2010), while N availability affected the signalling pathways related to Cd (Finkemeier *et al.*, 2003). Thus a further study is required, focusing mainly on the direct effect of Cd on the main enzymes of N metabolism, as well as transcription and expression of resistance genes related to Cd adaptation, to understand the mechanisms of sexual difference in metal sensitivity with differences in N status.

In conclusion, the present study presents an integrative research, including growth, physiological, biochemical, and ultrastructural responses to Cd and N deposition in females and males of *P. yunnanensis*. Cd stress alone induced more depression in biomass and gas exchange rate, greater damage to PSII, and higher disturbance of enzyme activities and redox homeostasis in females than in males. These results demonstrated that females are more sensitive to Cd, while males have a better detoxification mechanism to adapt to Cd stress. Such results are similar to those of preliminary experiments using cuttings cloned from a male and a female tree derived from a controlled intraspecific cross between two *P. yunnanensis* genotypes with distinct phenotypes, sampled from Meigu, as well as using female and male clones collected from a same population (data not shown), indicating that sexual differences of *P. yunnanensis* under Cd stress probably outweigh physiological differences derived from different genotypes and different populations. Such a discrepancy between the sexes in terms of stress sensitivity either might correlate with sex-related reproduction costs or could be related to sex-specific changes in metabolism and regulation. On the other hand, Cd toxicities are relieved in both sexes when N deposition is applied. It should be pointed out that sex-specific differences in response to Cd lessen under such conditions. Therefore, in practice, the soil N status can be modified artificially according to the background level of nutrients to improve Cd tolerance of *P. yunnanensis* and stimulate biomass accumulation simultaneously. In addition, males of *P. yunnanensis* could be promising candidates for phytoextraction and phytoremediation of Cd, especially in heavily contaminated areas, because of their good growth performance and tolerance under exposure to this metal.

Acknowledgements

The research was supported by the Key Program of the National Natural Science Foundation of China (no. 30930075).

References

Benavides MP, Gallego SM, Tomaro ML. 2005. Cadmium toxicity in plants. *Brazilian Journal of Plant Physiology* **17,** 21–34.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Brennan T, Frenkel C. 1977. Involvement of hydrogen peroxide in the regulation of senescence in pear. *Plant Physiology* **59,** 411–416.

Chaffei C, Pageau K, Suzuki A, Gouia H, Ghorbel MH, Masclaux-Daubresse C. 2004. Cadmium toxicity induced changes in nitrogen management in *Lycopersicon esculentum* leading to a metabolic safeguard through an amino acid storage strategy. *Plant and Cell Physiology* **45,** 1681–1693.

Chen LH, Zhang S, Zhao HX, Korpelainen H, Li CY. 2010. Sex-related adaptive responses to interaction of drought and salinity in *Populus yunnanensis*. *Plant Cell and Environment* **33,** 1767–1778.

Cobbett C, Goldsbrough P. 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology* **53,** 159–182.

Correia I, Nunes A, Duarte IF, Barros A, Delgadillo I. 2005. Sorghum fermentation followed by spectroscopic techniques. *Food Chemistry* **90,** 853–859.

Darwin C. 1877. *The different forms of flowers on plants of the same species*. London: John Murray, 278–309.

Dawson TE, Ehleringer JR. 1993. Gender-specific physiology, carbon isotope discrimination, and habitat distribution in boxelder. *Acer negundo. Ecology* **74,** 798–815.

Durand TC, Sergeant K, Planchon S, Carpin S, Label P, Morabito D, Hausman JF, Renaut J. 2010. Acute metal stress in *Populus tremula*× *P. alba* (717-1B4 genotype): leaf and cambial proteome changes induced by cadmium^{2+}. *Proteomics* **10,** 349–368.

Ehlting B, Dluzniewska P, Dietrich H, *et al*. 2007. Interaction of nitrogen nutrition and salinity in Grey poplar (*Populus tremula*× *alba*). *Plant, Cell and Environment* **30,** 796–811.

Ellman GL. 1959. Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics* **82,** 70–77.

Eppley SM. 2006. Females make tough neighbors: sex-specific competitive effects in seedlings of a dioecious grass. *Oecologia* **146,** 549–554.

Finkemeier I, Kluge C, Metwally A, Georgi M, Grotjohann N, Dietz KJ. 2003. Alterations in Cd-induced gene expression under nitrogen deficiency in *Hordeum vulgare*. *Plant, Cell and Environment* **26,** 821–833.

Garnier L, Simon-Plas F, Thuleau P, Agnel JP, Blein JP, Ranjeva R, Montillet JL. 2006. Cadmium affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity. *Plant, Cell and Environment* **29,** 1956–1969.

Hassan MJ, Wang F, Ali S, Zhang GP. 2005. Toxic effect of cadmium on rice as affected by nitrogen fertilizer form. *Plant and Soil* **277,** 359–365.

Hodges DM, DeLong JM, Forney CF, Prange RK. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* **207,** 604–611.

Kampfenkel K, Van Montagu M, Inzé D. 1995. Extraction and determination of ascorbate and dehydroascorbate from plant tissue. *Analytical Biochemistry* **225,** 165–167.

Kieffer P, Dommes J, Hoffmann L, Hausman JF, Renaut J. 2008. Quantitative changes in protein expression of cadmium-exposed poplar plants. *Proteomics* **8,** 2514–2530.

Kieffer P, Schröder P, Dommes J, Hoffmann L, Renaut J, Hausman JF. 2009. Proteomic and enzymatic response of poplar to cadmium stress. *Journal of Proteomics* **72,** 379–396.

Krupa Z, Baszynski T. 1995. Some aspects of heavy metals toxicity towards photosynthetic apparatus—direct and indirect effects on light and dark reactions. *Acta Physiologiae Plantarum* **17,** 177–190.

Küpper H, Kochian LV. 2010. Transcriptional regulation of metal transport genes and mineral nutrition during acclimatization to cadmium and zinc in the Cd/Zn hyperaccumulator. *Thlaspi caerulescens (Ganges population)New Phytologist* **185,** 114–129.

Küpper H, Parameswaran A, Leitenmaier B, Trtílek M, Šetlík I. 2007. Cadmium-induced inhibition of photosynthesis and long-term acclimation to cadmium stress in the hyperaccumulator. *Thlaspi caerulescens. New Phytologist* **175,** 655–674.

Lei YB, Yin CY, Li CY. 2006. Differences in some morphological, physiological, and biochemical responses to drought stress in two contrasting populations of *Populus przewalskii*. *Physiologia Plantarum* **127,** 182–191.

Li CY, Xu G, Zang RG, Korpelainen H, Berninger F. 2007. Sex-related differences in leaf morphological and physiological responses in *Hippophae rhamnoides* along an altitudinal gradient. *Tree Physiology* **27,** 399–406.

Li JY, Fu YL, Pike SM, *et al*. 2010. The *Arabidopsis* nitrate transporter NRT1.8 functions in nitrate removal from the xylem sap and mediates cadmium tolerance. *The Plant Cell* **22,** 1633–1646.

Loggini B, Scartazza A, Brugnoli E, Navari-Izzo F. 1999. Antioxidative defense system, pigment composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. *Plant Physiology* **119,** 1091–1099.

Maxwell K, Johnson GN. 2000. Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany* **51,** 659–668.

Mehta SK, Gaur JP. 1999. Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in. *Chlorella vulgaris. New Phytologist* **143,** 253–259.

Metwally A, Finkemeier I, Georgi M, Dietz KJ. 2003. Salicylic acid alleviates the cadmium toxicity in barley seedlings. *Plant Physiology* **132,** 272–281.

Metwally A, Safronova VI, Belimov AA, Dietz KJ. 2005. Genotypic variation of the response to cadmium toxicity in *Pisum sativum* L. *Journal of Experimental Botany* **56,** 167–178.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7,** 405–410.

Noctor G, Foyer CH. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology* **49,** 249–279.

Olmos E, Martínez-Solano JR, Piqueras A, Hellín E. 2003. Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line). *Journal of Experimental Botany* **54,** 291–301.

Panković D, Plesničar M, Arsenijević-Maksimović I, Petrović N, Sakač Z, Kastori R. 2000. Effects of nitrogen nutrition on photosynthesis in Cd-treated sunflower plants. *Annals of Botany* **86,** 841–847.

Perfus-Barbeoch L, Leonhardt N, Vavasseur A, Forestier C. 2002. Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. *The Plant Journal* **32,** 539–548.

Phoenix GK, Hicks WK, Cinderby S, *et al*. 2006. Atmospheric nitrogen deposition in world biodiversity hotspots: the need for a greater global perspective in assessing N deposition impacts. *Global Change Biology* **12,** 470–476.

Pietrini F, Iannelli MA, Pasqualini S, Massacci A. 2003. Interaction of cadmium with glutathione and photosynthesis in developing leaves and chloroplasts of *Phragmites australis* (Cav.) Trin. ex Steudel. *Plant Physiology* **133,** 829–837.

Pietrini F, Zacchini M, Iori V, Pietrosanti L, Ferretti M, Massacci A. 2010. Spatial distribution of cadmium in leaves and its impact on photosynthesis: examples of different strategies in willow and poplar clones. *Plant Biology* **12,** 355–363.

Rodríguez-Serrano M, Romero-Puertas MC, Pazmiño DM, Testillano PS, Risueño MC, del Río LA, Sandalio LM. 2009. Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. *Plant Physiology* **150,** 229–243.

Romero-Puertas MC, Corpas FJ, Rodríguez-Serrano M, Gómez M, del Río LA, Sandalio LM. 2007. Differential expression and regulation of antioxidative enzymes by cadmium in pea plants. *Journal of Plant Physiology* **164,** 1346–1357.

Romero-Puertas MC, Palma JM, Gómez M, Del Río LA, Sandalio LM. 2002. Cadmium causes the oxidative modification of proteins in pea plants. *Plant, Cell and Environment* **25,** 677–686.

Sandalio LM, Dalurzo HC, Gómez M, Romero-Puertas MC, del Río LA. 2001. Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *Journal of Experimental Botany* **52,** 2115–2126.

Saneoka H, Moghaieb REA, Premachandra GS, Fujita K. 2004. Nitrogen nutrition and water stress effects on cell membrane stability and leaf water relations in *Agrostis palustris* Huds. *Environmental and Experimental Botany* **52,** 131–138.

Schützendübel A, Schwanz P, Teichmann T, Gross K, Langenfeld-Heyser R, Godbold DL, Polle A. 2001. Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots pine roots. *Plant Physiology* **127,** 887–898.

Sharma SS, Dietz KJ. 2006. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *Journal of Experimental Botany* **57,** 711–726.

Sharma SS, Dietz KJ. 2009. The relationship between metal toxicity and cellular redox imbalance. *Trends in Plant Science* **14,** 43–50.

Sigfridsson KGV, Bernát G, Mamedov F, Styring S. 2004. Molecular interference of Cd^{2+} with photosystem II.. *Biochimica et Biophysica Acta* **1659,** 19–31.

Siripornadulsil S, Traina S, Verma DPS, Sayre RT. 2002. Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. *The Plant Cell* **14,** 2837–2847.

Solti Á Gáspár L, Mészáros I, Szigeti Z, Lévai L, Sárvári É. 2008. Impact of iron supply on the kinetics of recovery of photosynthesis in Cd-stressed poplar (*Populus glauca*). *Annals of Botany* **102,** 771–782.

Stevens CJ, Dise NB, Mountford JO, Gowing DJ. 2004. Impact of nitrogen deposition on the species richness of grasslands. *Science* **303,** 1876–1879.

Utriainen J, Holopainen T. 2001. Nitrogen availability modifies the ozone responses of Scots pine seedlings exposed in an open-field system. *Tree Physiology* **21,** 1205–1213.

Van Assche F, Clijsters H. 1990. Effects of metals on enzyme activity in plants. *Plant, Cell and Environment* **13,** 195–206.

van Kooten O, Snel JFH. 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynthesis Research* **25,** 147–150.

Vögeli-Lange R, Wagner GJ. 1990. Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves: implication of a transport function for cadmium-binding peptides. *Plant Physiology* **92,** 1086–1093.

Wang L, Zhou QX, Ding LL, Sun YB. 2008. Effect of cadmium toxicity on nitrogen metabolism in leaves of *Solanum nigrum* L. as a newly found cadmium hyperaccumulator. *Journal of Hazardous Materials* **154,** 818–825.

Wawrzyński A, Kopera E, Wawrzyńska A, Kamińska J, Bal W, Sirko A. 2006. Effects of simultaneous expression of heterologous genes involved in phytochelatin biosynthesis on thiol content and cadmium accumulation in tobacco plants. *Journal of Experimental Botany* **57,** 2173–2182.

Xie HL, Jiang RF, Zhang FS, McGrath SP, Zhao FJ. 2009. Effect of nitrogen form on the rhizosphere dynamics and uptake of cadmium and zinc by the hyperaccumulator. *Thlaspi caerulescens. Plant and Soil* **318,** 205–215.

Xu X, Yang F, Xiao XW, Zhang S, Korpelainen H, Li CY. 2008. Sex-specific responses of *Populus cathayana* to drought and elevated temperatures. *Plant, Cell and Environment* **31,** 850–860.

Yao XQ, Liu Q. 2007. Changes in photosynthesis and antioxidant defenses of *Picea asperata* seedlings to enhanced ultraviolet-B and to nitrogen supply. *Physiologia Plantarum* **129,** 364–374.

Zhang S, Chen FG, Peng SM, Ma WJ, Korpelainen H, Li CY. 2010. Comparative physiological, ultrastructural and proteomic analyses reveal sexual differences in the responses of *Populus cathayana* under drought stress. *Proteomics* **10,** 2661–2677.

Zhang S, Jiang H, Peng SM, Korpelainen H, Li CY. 2011. Sex-related differences in morphological, physiological, and ultrastructural responses of *Populus cathayana* to chilling. *Journal of Experimental Botany* **62,** 675–686.

Zhao HX, Li Y, Duan BL, Korpelainen H, Li CY. 2009. Sex-related adaptive responses of *Populus cathayana* to photoperiod transitions. *Plant, Cell and Environment* **32,** 1401–1411.