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Phosphorus influence Cd phytoextraction in *Populus* stems via modulating xylem development, cell wall Cd storage and antioxidant defense

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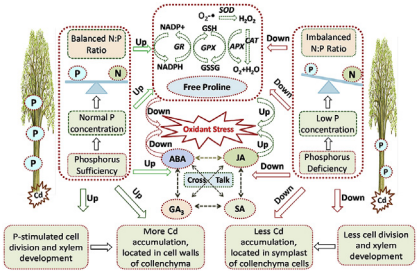
HIGHLIGHTS

- Sufficient tissue P concentration and balanced N:P ratio enhanced cell division in stem.
- Low tissue P concentration and imbalanced N:P ratio inhibited xylem development.
- Phosphorus increased cell wall affinity for Cd and Cd accumulation in cell wall in stem.
- P deficiency decreased phytohormone levels and increased oxidative stress in stem.
- Phosphorus enhanced Cd phytoextraction via promoting xylem development and antioxidant defense.

GRAPHICAL ABSTRACT



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ABSTRACT

The soils in mining lands with cadmium (Cd) contamination usually are deficient in nutrients. Disclosing how P nutrition and N:P stoichiometric ratio influences Cd accumulation and stress tolerance in stems of *Populus* spp. will facilitate the phytoremediation of mining sites polluted by Cd. In this study, investigations at the anatomical and physiological levels were conducted using a clone of *Populus* × *euramericana*. Both phosphorus deficiency and cadmium exposure inhibited xylem development via reducing cell layers in the xylem. Under P-sufficient condition, appropriate P status and balanced N:P ratio in stem promoted xylem development under Cd exposure via stimulating cell division, which enhanced Cd accumulation in stems. Cd accumulation in cell walls of collenchyma tissues of the stem was enhanced by P application due to increased polysaccharide production and cell wall affinity for Cd. The low P concentrations (0.3–0.4 mg g^{-1}) and imbalanced N:P ratio under P deficiency inhibited the production of APX and ascorbate–GSH cycle, which increased oxidative stress and lipid peroxidation as indicated by high MDA concentration in stem. Under P-sufficient condition, the interactions between

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N:P stoichiometric ratio
Populus

phytohormones and antioxidants play crucial roles in the process of antioxidant defense under Cd exposure. In conclusions, appropriate P addition and balanced N:P ratio enhanced secondary xylem development and promoted cadmium accumulation and stress tolerance in *Populus* stems, which can benefit the phytoextraction of Cd from Cd-contaminated soil.

1. Introduction

Environmental contamination by toxic heavy metals is increasing due to increasing human activities such as mining (He et al., 2013, 2015; Shi et al., 2015). Long-term mining is a major resource of heavy metal contamination and health risk to humans (Liu et al., 2014). Mining activities in China alone have generated about 3.0 million ha derelict land by the year 2000, and it is increasing at a rate of 46,700 ha per year (Li, 2006). The food security has been challenged dramatically with the loss of the arable lands in China (He et al., 2011; Liu et al., 2014). Therefore, ecological restoration of mining sites and phytoremediation of heavy metal contamination is critical to reduce health risk and enhance food security in China. After metal mining, the mining lands usually have inferior soil properties that are unfavorable to plant establishment, such as the deficiency of nutrients, poor physical structure and extreme pH (Li, 2006; Liu et al., 2014; Rees et al., 2016). To break through the constraints of nutrient deficiency to ecological restoration of mining sites with cadmium contamination, it is necessary to elucidate the mechanisms underlying the impact of nutrient status on cadmium accumulation and stress tolerance in plant species that are used for ecological restoration (Hacke et al., 2010; Zhang et al., 2019).

Cadmium (Cd) is a widespread heavy metal in mining lands that lead to pollution of soils and waters, and endanger the health of humans (Luo et al., 2013; Shi et al., 2015). Cd in the soil can be extracted by metal-tolerant plant species through absorption by roots, sequestering into the vacuole as Cd-phytochelatin complex, and translocation into shoots (Farooq et al., 2013; Chen et al., 2016; Cheng et al., 2017). Phosphorus (P) is an essential macronutrient which not only support plant growth but also reduce Cd toxicity via chelating or forming complex with Cd in plants, which may alleviate the damage of Cd to cell functions (Jiang et al., 2007; Gomes et al., 2014; Dai et al., 2017a). P addition decreased malonaldehyde via positively regulating the synthesis of glutathione and phytochelatins in mangrove plants (Dai et al., 2017a). The growth of *Mirabilis jalapa* L increased with P supplies under Cd exposure, and the translocation factor of Cd reached the maximum at 100 mg kg^{-1} P application (Dai et al., 2017a). However, the anatomical and physiological mechanisms underlying the influence of phosphorus on Cd accumulation and antioxidant defense in plant species with high Cd tolerance and large capacity for Cd phytoextraction are still not fully elucidated.

Cadmium hyperaccumulator plants have been proposed for the phytoremediation of Cd-contaminated mining sites as they are highly tolerant to this toxic metal and can absorb and accumulate large concentration of Cd from contaminated soils (McGrath et al., 2006; Liu et al., 2017; Wu et al., 2018). Some fast-growing woody species such as *Populus* spp. also showed high tolerance to Cd exposure (Chen et al., 2011; He et al., 2013, 2015; Shi et al., 2015). Although the tissue concentration of Cd in these woody species may be lower than herbaceous hyperaccumulators, some *Populus* species usually extract a similar or even higher total amount of Cd from contaminated soil due to their gigantic root system and great biomass (Wu et al., 2010; Chen et al., 2011; Zhang et al., 2019; Wang et al., 2019). To alleviate oxidative stress and avoid damages caused by Cd exposure, *Populus* species usually exhibit various adaptations such as the inducement of enzymatic and non-enzymatic antioxidants that are critical to the homeostasis of ROS production and scavenging (Seth et al., 2012; Shi et al., 2015; Redovniković et al., 2017; Zhang et al., 2019; Wang et al., 2019).

Once Cd ions are in the xylem of the roots, they pass up the xylem of the stem and transported to the leaves with the transpiration stream, or be re-distributed via phloem transport (He et al., 2015; Wang et al., 2019). The xylem-mediated Cd translocation into shoots is a major factor determining Cd accumulation in the shoots of many plants, and the disruption or wound of xylem may results in reduced Cd accumulation in above-ground tissues (Qin et al., 2013; Wang et al., 2019). There was an 80% reduction in stem diameter *Eucalyptus grandis* with decreasing P supply, all of which can be directly attributable to the reduction in cell numbers, as new cells require P for nucleic acids, membranes and energy supply during construction (Thomas et al., 2006). Previous research demonstrated that poplar species might respond to P deficiency by decreasing P uptake, mobilization and assimilation (Gan et al., 2016). Together with nitrogen, phosphorus is a key component of the metabolites involved in photosynthesis and the production of carbohydrates as well as antioxidants that are critical to sustaining antioxidative processes (Jiang et al., 2007; Rennenberg and Herschbach, 2013; Cheng et al., 2018). Moreover, plant growth is determined by interactions among nutrients, particularly N:P ratios in ecosystems and plant tissues, i.e. the relative availability of environmental N and P (Hu et al., 2016; Shi et al., 2017). Therefore, disclosing how P nutritional status and N:P stoichiometric ratio influences stem development and antioxidant defense, and finally affecting Cd accumulation in stems of *Populus* spp. will facilitate the phytoremediation of mining sites polluted by heavy metals.

In this study, investigations at the anatomical and physiological levels were conducted using a clone of *Populus* × *euramericana* to address the following hypotheses: (1) P status and N:P stoichiometric ratio will influence the secondary xylem development and the production of enzymatic and non-enzymatic antioxidants in stems of poplar under combined conditions of P and Cd. (2) P nutritional status and N:P stoichiometric ratio will influence Cd accumulation in stems via regulating the secondary xylem development and antioxidant defense. This study will provide important plant nutritional guidelines for the phytoremediation of mining sites polluted by heavy metals using poplar species.

2. Materials and methods

2.1. Plant material

A hybrid poplar species, *Populus* × *euramericana* which showed favorable tolerance to heavy metal, drought and salt stress (Wu et al., 2010; Di Baccio et al., 2011) was used as the plant material. Plantlets were produced by micropropagation and cultivated in a climate chamber (day/night temperature, 25/18 °C; relative air humidity, 50–60%; light per day, 14 h; photosynthetic photon flux, 100 μmol m^{-2} s^{-1}). After growing for 5 weeks, rooted plantlets

were transferred to 10-L plastic pots filled with fine sand. Subsequently, plants were grown for 4 weeks in a greenhouse (day/night temperature, 25/18 °C; relative air humidity, 50–60%; light per day, natural light). After growing for 4 weeks, plants with similar height were selected and divided into several groups for further treatments.

2.2. Experimental design

A factorial design consisting of P and Cd treatments was applied. There were three Cd levels and two P levels in the factorial design. The three Cd levels were 0 mg Cd kg^{-1} sand, 4 mg Cd kg^{-1} sand and 20 mg Cd kg^{-1} sand respectively, which were achieved by adding 0, 7.4, or 37.0 μg g^{-1} $CdSO_4$ into the sand in the form of a solution. Modified LA solution (Luo et al., 2013) with normal P and deficient P nutrient was applied to pots of the control and the deficient-P treatment, respectively. Phosphorus concentrations in deficient P treatment (0.06 mM KH_2PO_4, 0.0042 mM K_2HPO_4) was equal to 10% of phosphorus concentrations in the control (0.6 mM KH_2PO_4, 0.042 mM K_2HPO_4), while the nutrient concentrations except P (1.0 mM NH_4NO_3, 0.5 mM KCl, 0.9 mM $CaCl_2$, 0.3 mM $MgSO_4$, 10 μM Fe-EDTA, 2 μM $MnSO_4$, 10 μM H_3BO_3, 7 μM Na_2MoO_4, 0.05 μM $CoSO_4$, 0.2 μM $ZnSO_4$, and 0.2 μM $CuSO_4$) were same for the control and low P treatments. Although deficient P treatment had a low concentration (0.06 mM) of KH_2PO_4, the plants in deficient P treatment can obtain adequate K^+ as 0.5 mM KCl was equally applied in both P treatments. Therefore, the influence of K^+ deficiency on Cd accumulation and Cd tolerance can be neglected. The nutrient solution was applied at a rate of 200 mL per pot every day. There were 9 replicates in each treatment, giving a total of 54 seedlings. The P and Cd treatments were applied for 60 days until harvest. The total time of plantlets growth was 88 days.

2.3. Measurements of seedling growth traits and tissue P and N concentration

At harvest, plant height, stem basal diameter and fresh weight of each organ were measured and then frozen immediately in liquid nitrogen. The frozen samples were ground into a fine powder, during which the liquid nitrogen was applied to maintain the freezing status. The fine powder of each sample was stored at −80 °C until further analysis. The fresh: dry mass ratio were determined via drying the fresh sample (about 100 mg) at 65 °C for 72 h and measuring the dry biomass (Gan et al., 2016). The biomass of each organ was determined using the fresh weight and the fresh: dry mass ratio. Root to shoot ratios (R/S) was calculated as the ratio of root biomass to shoot biomass. To determine the concentrations of P and N in each organ, *approx.* 100 mg sample were digested, and subsequently, the P concentration and N concentration in the digested solution was determined according to the method described by previous reports (Gan et al., 2016).

2.4. Determination of tissue distribution of Cd and anatomical features of stem

To determine the patterns of localization and distribution Cd in stems, histochemical staining of Cd was conducted using fresh samples from poplar seedlings grown under high Cd treatment (20 mg Cd kg^{-1} soil) and control (0 mg Cd kg^{-1} soil). Sections of the stems (ca. 10 cm long below the top of stem) were rinsed in de-ionized H_2O. Subsequently, fresh samples of stems were immersed in a staining solution for 1 h and then rinsed briefly in de-ionized H_2O (He et al., 2015). Subsequently, the well-stained sections with Cd-dithizone precipitates were photographed under a light microscope (Eclipse E200; Nikon, Tokyo, Japan) using a CCD (DS-Fi1; Nikon) connected to a computer, as described by previous reports (He et al., 2015). The stained sections of stems were photographed to perform anatomical analysis. Fiber lumen diameters, xylem cell layers, and thicknesses of xylem and phloem were quantified using the method described by previous studies (Song et al., 2019).

2.5. Determination of Cd accumulation and Cd bioconcentration factor in stems

To measure Cd content in stems, fine powder (ca. 100 mg) of samples was digested at 170 °C in a mixture of acid (7 ml HNO_3 and 1 ml concentrated $HClO_4$). Subsequently, Cd was determined by flame atomic absorbance spectrometry (Hitachi 180–80, Japan). Cadmium concentration was measured using flame atomic absorbance spectrometry (Hitachi 180–80; Hitachi Ltd, Tokyo, Japan) according to the reported method (He et al., 2015). Cadmium content was calculated by multiplying the Cd concentration in each organ by the organ biomass. Bioconcentration factor (BCF) was calculated as the ratio of Cd concentration in an organ to the Cd concentration in the soil (He et al., 2013).

2.6. Measurement of the N and P concentration and N:P stoichiometric ratio

For the measurement of physiological parameters related to stress tolerance, fresh samples from three seedlings were incorporated to generate a mixed sample, resulting in three biological replicates in each treatment which consisting of 9 seedlings. The dried plant organs were used for measurement of the N and P concentration, using a method described by Gan et al. (2016). Fine powder (~100 mg DW) was digested by 5 ml 98% H_2SO_4 and 1 ml H_2O_2. The P concentration was determined spectrophotometrically at 700 nm based on the molybdenum blue method. The N concentrations were analyzed using a Continuous-Flow Analyzer (AA3, Bran-Luebbe, Hamburg, Germany) at 660 nm. The N:P stoichiometric ratio was calculated as tissue N concentration divided by tissue P concentration.

2.7. Physiological parameters related to stress tolerance in stems

The concentration of soluble sugars was determined using the previous method (Luo et al., 2013). The concentration of free proline was determined using the following method. Fresh samples (ca. 100 mg) was extracted by 1.5 ml of 3% sulfosalicylic acid at 100 °C for 15 min, and then they were centrifugated for 10 min at 12,000 g. Subsequently, 1 mL glacial acetic acid and 1 mL ninhydrin reagent was mixed with the supernatant and then incubated at 98 °C for 30 min. Finally, the solution were spectrophotometrically measured at 518 nm. Using methods reported by Chen et al. (2011), the activities of antioxidant enzymes were determined, which including peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; E.C. 1.11.1.6) and glutathione reductase (GR; EC 1.6.4.2). Malonaldehyde (MDA) concentration was determined based on the method previously reported (He et al., 2013).

Phytohormone levels in stems including jasmonic acid (JA), abscisic acid (ABA), gibberellic acid (GA_3), and salicylic acid (SA) were analyzed through high-performance liquid chromatography-electrospray ionization-ion trap mass spectrometry. Briefly, 4 mL of 80% (v/v) methanol containing 200 mg μL^{-1} of butylated hydroxytoluene and 500 mg L^{-1} of citric acid monohydrate was used to extract phytohormones from the powder of fresh samples (ca. 500 mg). The extracting solution was shaken at 4 °C for 12 h, and then centrifugated at 10,000 g and 4 °C for 15 min. The supernatant was collected, and the precipitate was extracted twice using the

same method. The supernatants were incorporated and dried by N_2. Before measurement, the dried samples were re-suspended by 800 μL of 80% methanol, and were analyzed by an LC-20AT high-performance liquid chromatography system (Shimadzu, Kinh Do, Japan) combined with an API 2000™ electrospray tandem mass spectrometer (Allen-Bradley, Milwaukee, WI, USA). ABA [(±)-ABA, A1049], GA_3 (G7645), SA (S7401), and IAA (I2886) were used to make standard curves.

2.8. Statistical analysis

The normality of data was tested before statistical analyses using the UNIVARIATE procedure of SAS software (SAS Institute, Cary, NC; 1996). Statistical tests were performed with SAS software (SAS Institute, Cary, NC; 1996). The *P*-values of multiple comparisons were calculated using Tukey's HSD method to avoid type I errors (He et al., 2015). Differences between values were considered to be significant when the *P*-value of the ANOVA *F*-test was <0.05. Linear correlations were analyzed by the CORR procedure in the SAS software (SAS Institute, Cary, NC; 1996), and were considered to be significant when the *P*-value was less than 0.05. The principal component analysis (PCA) were performed by the command prcomp () in R (http://www.rproject.org/) as reported previously (Zheng et al., 2017).

3. Results

3.1. Biomass and anatomical features of stem

Plant biomass, plant height, stem basal diameter and stem biomass were decreased by P deficiency regardless of Cd treatments, while root biomass was unaltered by P status (Fig. 1). As a result, R/S was increased by P deficiency. Plant biomass and stem biomass were decreased by Cd exposure under both deficient- and normal- P conditions, while root biomass was unaltered by Cd exposure. R/S increased upon Cd exposure under both P conditions (Fig. 1). The degree of suppression on plant biomass and stem biomass was the largest under the combination of Cd exposure and P deficiency (Fig. 1).

Phosphorus and cadmium had significant effects on xylem thickness and xylem cell layers. Cd exposure suppressed xylem development under both P deficiency and control, resulting in the lower thickness and less cell layers of xylem (Fig. 2 a-d; Fig. 2 i-j). Xylem development was suppressed by P deficiency under high or zero Cd exposure. The degree of suppression on xylem development was the largest under the combination of Cd exposure and P

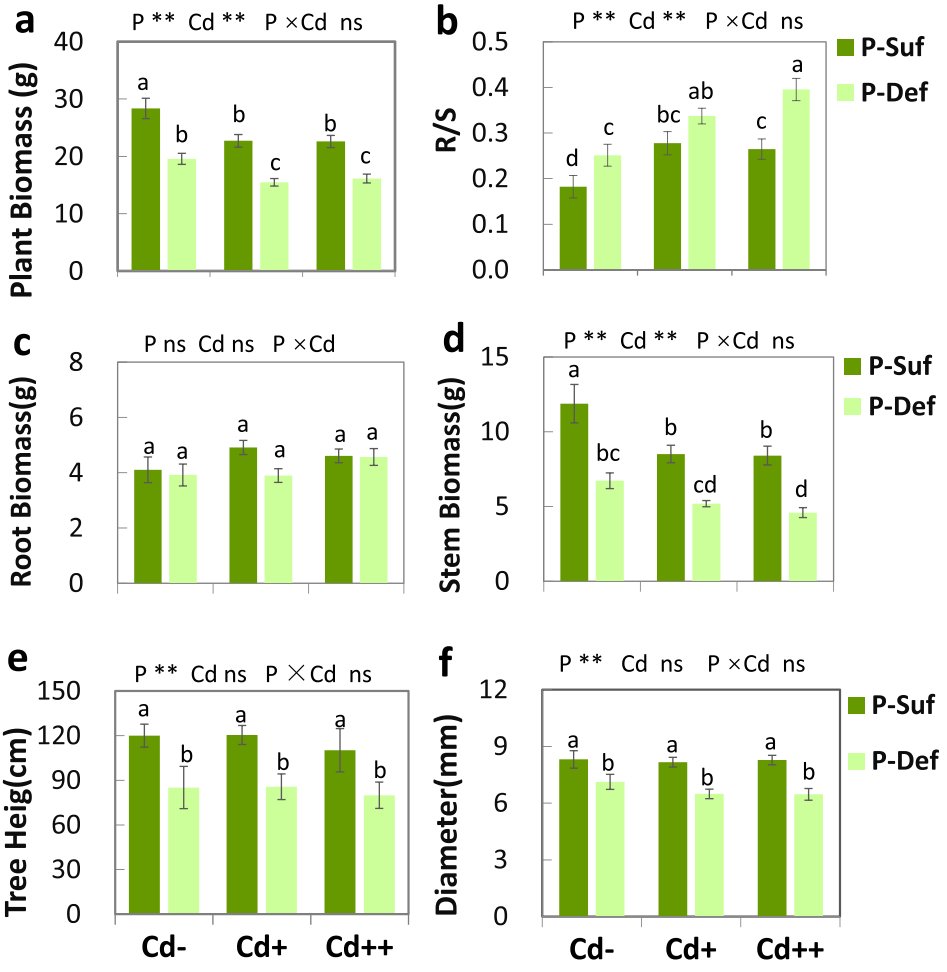

Fig. 1. Plant biomass (a), R/S (b), root biomass (c) and stem biomass (d) of *Populus euramericana* under combined phosphorus and cadmium treatments. P-Suf, P-sufficient condition (0.6 mM KH_2PO4, 0.042 mM K_2HPO4); P-Def, P-deficient condition with 10% of phosphorus addition in the P-sufficient condition (0.06 mM KH_2PO4, 0.0042 mM K_2HPO4); Cd-, no added Cd; Cd+, 4.0 mg kg^{-1}; Cd++, 20 mg kg^{-1}; The bars indicate means ± SE (n = 12). Different letters on the bars indicate significant differences in ANOVA ($P < 0.05$). *P* values from two-way ANOVA on phosphorus (P), cadmium (Cd) and their interaction (P × Cd) are indicated (*, $P < 0.05$; **, $P < 0.01$; ns, not significant) above each panel.

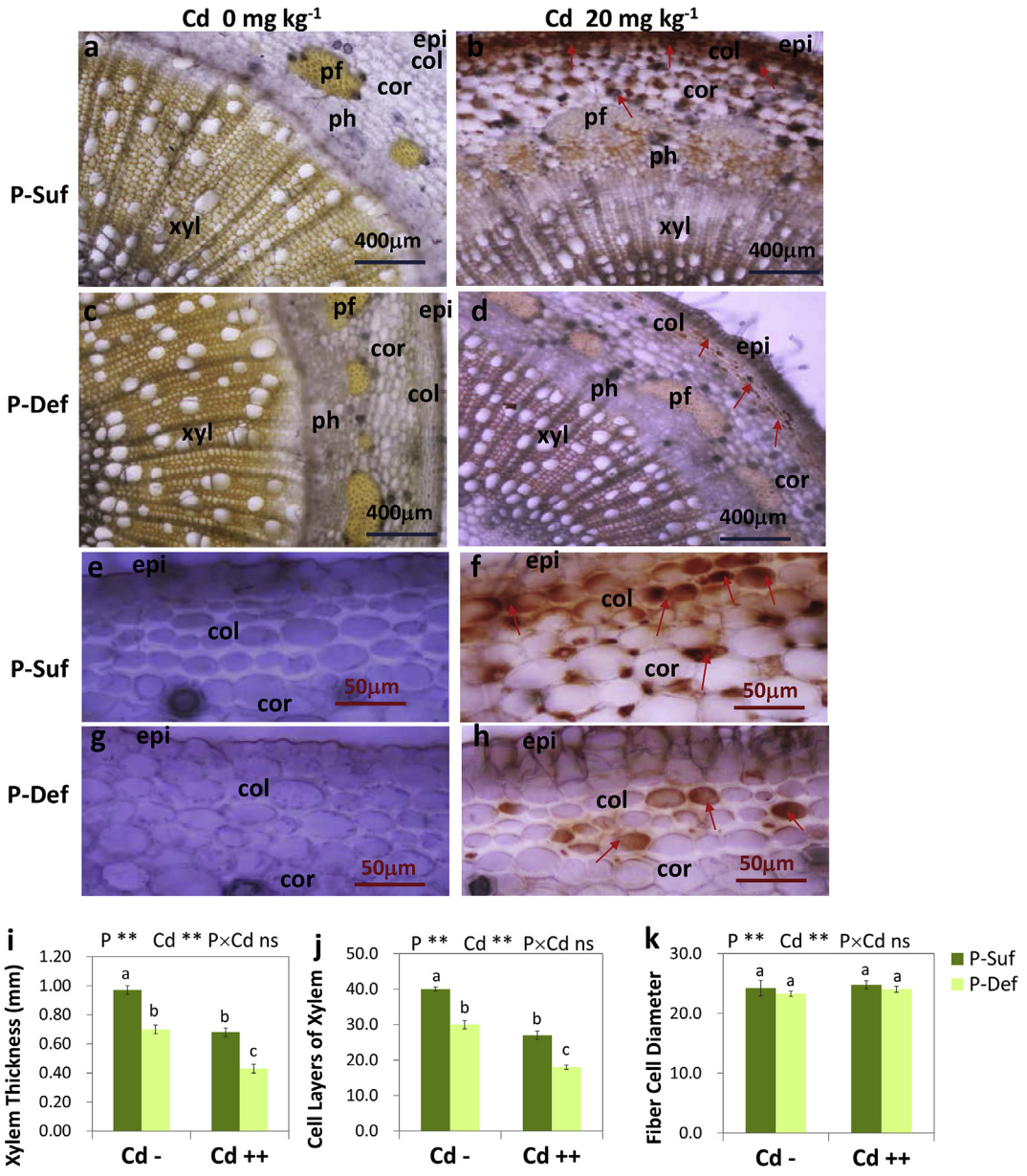

Fig. 2. Cadmium localization (a–h) and anatomical properties (i–k) in stems of *Populus euramericana* under combined treatments of P and Cd. (a) and (e), P sufficient condition and zero Cd exposure (0 mg kg^{-1}). (b) and (f), P sufficient condition and high Cd exposure (20 mg kg^{-1}). (c) and (g), P deficient condition and zero Cd exposure (0 mg kg^{-1}). (d) and (h), P deficient condition and high Cd exposure (20 mg kg^{-1}) Blue bars = 200 μm. Red bars = 50 μm. Red arrows point to Cd dithizone precipitate. epi, epidermis; col, collenchyma; cor, cortex; ph, pholem; pf, phloem fiber; xyl, xylem. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

deficiency, while the suppression was partially alleviated by P application (Fig. 2) (Fig. 2 a-d; Fig. 2 i-j). The suppression on xylem development was a result of decreased cell layers of xylem but not fiber cell size, as fiber size was unaltered by P deficiency or Cd exposure (Fig. 2 i-j).

3.2. Accumulation and bioconcentration of Cd and its correlation with xylem development

In stems, Cd was located mainly in the symplast of collenchyma cells (Fig. 2h) when soil P was deficient. In contrast, Cd was distributed more widely and intensely under normal P condition than that of low P status, and it was located in symplast and cell walls of collenchyma cells, as well as in apoplast and intercellular space of cortical cells (Fig. 2f). The concentration of Cd in stems were decreased by P deficiency, which decrased from *approx.* 12 μg mg^{-1} to 4 μg mg^{-1}, and from *approx.* 18 μg mg^{-1} to 12 μg mg^{-1} under moderate and high Cd exposure, respectively (Fig. 3). As a result, Cd content in stem decreased from *approx.* 120 μg $plant^{-1}$ to 20 μg $plant^{-1}$, and from *approx.* 160 μg $plant^{-1}$ to 60 μg $plant^{-1}$ under moderate and high Cd exposure, respectively (Fig. 3). The bioconcentration factor (BCF) of Cd in stems was decreased by P deficiency from *approx.* 3.0 to 1.0 under moderate Cd expsure, while the changes by P application was not significant under high Cd exposure (Fig. 3). Under high Cd exposure, the concentration of Cd in stems was positively correlated with the xylem thickness and the number of cell layers, with the coefficient of determination (R^2) being 0.84 and 0.75, respectively (Fig. S1).

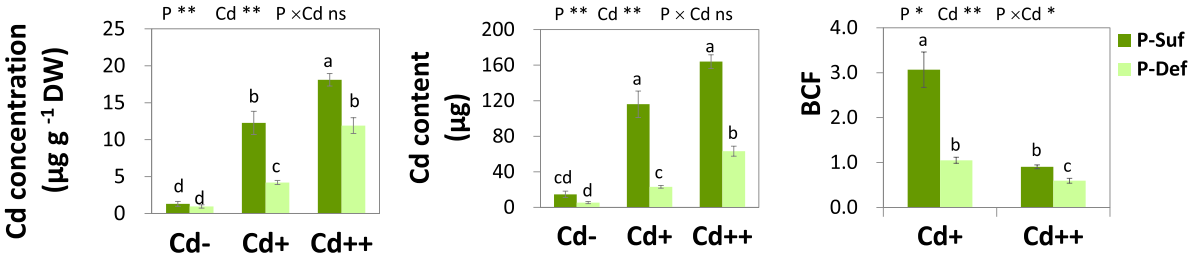

Fig. 3. Cadmium concentration, content and bioconcentration factor in stems. P-Suf, P-sufficient condition (0.6 mM KH_2PO4, 0.042 mM K_2HPO4); P-Def, P-deficient condition with 10% of phosphorus addition in the P-sufficient condition (0.06 mM KH_2PO4, 0.0042 mM K_2HPO4); Cd-, no added Cd; Cd +, 4 mg kg^{-1}; Cd++, 20 mg kg^{-1}; The bars indicate means ± SE (n = 4). Different letters above the bars indicate significant differences from ANOVA ($P < 0.05$). *P*-values from two-way ANOVA on phosphorus (P), cadmium (Cd) and their interaction (N × Cd) are indicated (*, $P < 0.05$; **, $P < 0.01$; ns, not significant) above each panel.

3.3. Phosphorus status, N:P ratios and their correlations with Cd accumulation and xylem development

In stems, P concentrations sharply decreased upon soil P deficiency regardless of Cd level, decreased from around 1.8–2.0 mg g^{-1} under normal P condition to around 0.3–0.4 mg g^{-1} under P deficiency. In comparison, tissue N concentrations were not altered by soil P conditions at each Cd level (Table 1). As a result, tissue N: P ratios in stems sharply increased from around 4.5–6.5 under normal P condition to around 17.0–23.0 under P deficiency (Table 1). Under both P conditions, concentrations of P, N and N: P ratios in stems all were unaltered by Cd exposure (Table 1).

Under high Cd exposure, P concentration in both roots and stems had a positive correlation with xylem thickness and cell layers in xylem (Table S1). In contrast, N:P ratios in both roots and stems had a negative correlation with the thickness and cell layers of xylem. Phosphorus concentration in both roots and stems had a strong and positive correlation with the concentration and content of Cd in stem. The trend of N: P ratio was opposite to that of P concentration, as N: P ratio in both stems and roots are negatively correlated with Cd concentration and Cd content in stems (Table S1).

3.4. Concentrations of free proline, soluble sugars and soluble protein in stems

Under normal P conditions, the level of free proline in stems was increased by a high or moderate level of Cd exposure as compared with zero Cd exposure (Table S2). In low-P treatment, the levels of free proline, soluble sugars and soluble proteins in stems were all unaltered by Cd exposure. Under the high or moderate level of Cd exposure, the level of free proline in stems decreased upon P deficiency (Table S2). The levels of soluble sugars and soluble protein were unaltered by P deficiency at each Cd level,.

3.5. The levels of antioxidant enzymes, GSH and MDA in stems

Under normal P condition, GSH concentration and the activities of APX and POD all increased upon high Cd exposure, while all antioxidant enzymes were unaltered by moderate Cd exposure (Fig. 4; Fig. S2). Under P deficient condition, POD was stimulated by both moderate- and high- Cd exposure (Fig. 4), while GR and CAT activity increased upon moderate- and high- Cd exposure, respectively. GR and CAT activity increased upon P deficiency at a moderate and high level of Cd exposure, respectively. The activities of the other enzymes were unaltered by P status regardless of Cd level (Fig. 4).

MDA level in the stems was higher under low P conditions than that of control at each Cd level. High Cd exposure increased MDA level under deficient P condition, but not under normal P condition (Fig. S3). MDA concentration in stem is negatively correlated with xylem thickness ($R^2 = 0.79$) and the cell layers of xylem ($R^2 = 0.81$) (Fig. S4).

3.6. The levels of phytohormones in stems

Under normal P condition, ABA was unaltered by Cd exposure, while JA was decreased by moderate or high level of Cd exposure. In comparison, GA_3 was increased by both moderate- and high- Cd exposure, and SA was increased by high Cd exposure (Fig. 5). Under P-deficient condition, all of the tested phytohormones (ABA, JA, GA_3 and SA) were decreased by a high level of Cd exposure, and they were also decreased by moderate Cd exposure except SA (Fig. 5).

The levels of ABA, GA_3 and SA were increased by P deficiency in the absence of Cd exposure. In contrast, the levels of JA and SA decreased upon P deficiency under moderate- and high Cd

Table 1
Tissue concentrations of P, N and N: P ratio in stems.

Phosphorus	Cadmium	P (mg g^{-1})	N (mg g^{-1})	N: P Ratio
Sufficient-P	Cd 0 mg kg^{-1}	2.0 ± 0.2 a	9.5 ± 1.9 a	4.5 ± 0.7 b
	Cd 4 mg kg^{-1}	1.8 ± 0.1 a	8.5 ± 0.6 a	4.9 ± 0.4 b
	Cd 20 mg kg^{-1}	1.9 ± 0.2 a	8.7 ± 1.2 a	4.7 ± 0.4 b
Deficient-P	Cd 0 mg kg^{-1}	0.3 ± 0.04 b	7.3 ± 0.6 a	23.1 ± 3.2 a
	Cd 4 mg kg^{-1}	0.4 ± 0.03 b	7.8 ± 0.5 a	21.5 ± 1.1 a
	Cd 20 mg kg^{-1}	0.4 ± 0.04 b	7.5 ± 0.8 a	22.4 ± 1.8 a
F Value of Two-way ANOVA	Phosphorus (P)	202.8**	2.7	179.1**
	Cadmium (Cd)	0.3	0.04	0.07
	P × Cd	0.4	0.3	0.2

The values are means ± SE (n = 9). Means with different letters are statistically different in one-way ANOVA ($P < 0.05$). *F* Value of two-way ANOVA (phosphorus and cadmium) are indicated (*, $P < 0.05$; **, $P < 0.01$).

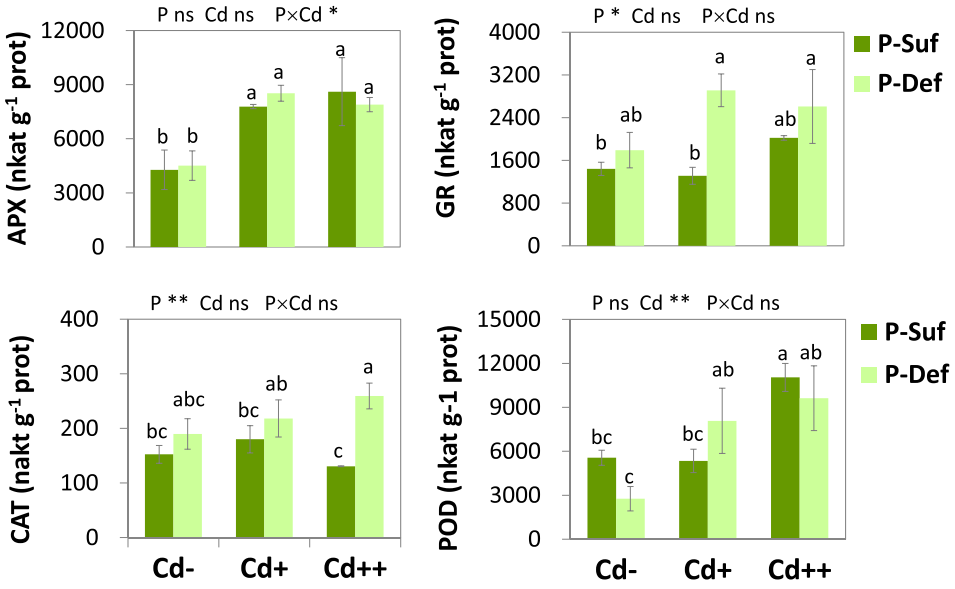

Fig. 4. Activity of antioxidant enzymes in stems. APX, ascorbate peroxidase. GR, glutathione reductase. CAT, catalase. POD, peroxidase. P-Suf, P-sufficient condition (0.6 mM KH_2PO4, 0.042 mM K_2HPO4); P-Def, P-deficient condition with 10% of phosphorus addition in the P-sufficient condition (0.06 mM KH_2PO4, 0.0042 mM K_2HPO4); Cd-, no added Cd; Cd +, 4 mg kg^{-1}; Cd ++, 20 mg kg^{-1}; The bars indicate means ± SE (n = 4). Different letters above the bars indicate significant differences by ANOVA ($P < 0.05$). *P*-values of two-way ANOVA on phosphorus (P), cadmium (Cd) and their interaction (N × Cd) are indicated above each panel (*, $P < 0.05$; **, $P < 0.01$; ns, not significant).

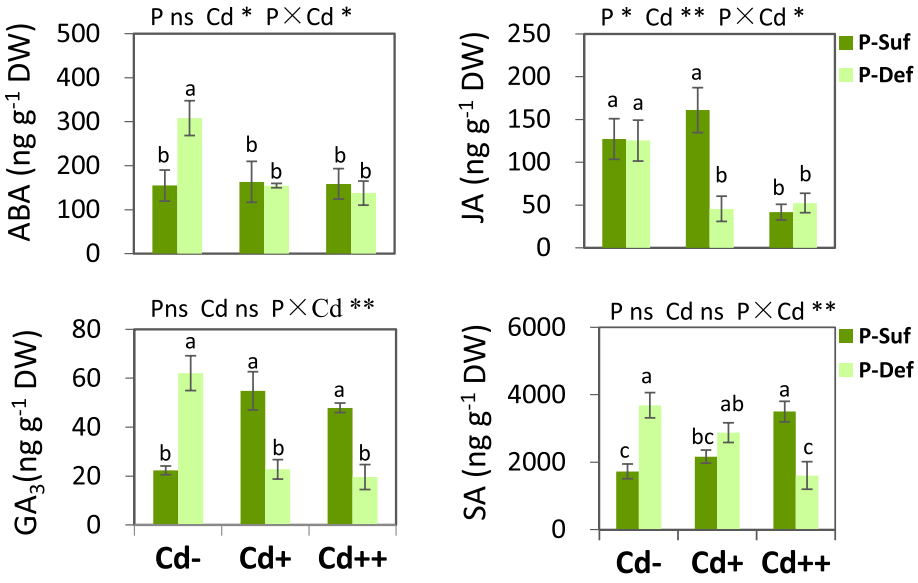

Fig. 5. Concentrations of phytohormones in stems. ABA, abscisic acid. JA, jasmonic acid. SA, salicylic acid. GA, gibberellic acid. P-Suf, P-sufficient condition (0.6 mM KH_2PO4, 0.042 mM K_2HPO4); P-Def, P-deficient condition with 10% of phosphorus addition in the P-sufficient condition (0.06 mM KH_2PO4, 0.0042 mM K_2HPO4); Cd-, no added Cd; Cd+, 4 mg kg^{-1}; Cd++, 20 mg kg^{-1}; The bars indicate means ± SE (n = 4). Different letters above the bars indicate significant differences by ANOVA ($P < 0.05$). *P*-values of two-way ANOVA on phosphorus (P), cadmium (Cd) and their interactions (N × Cd) are indicated above each panel (*, $P < 0.05$; **, $P < 0.01$; ns, not significant).

exposure, respectively. The levels of GA_3 decreased upon P deficiency at both Cd exposure levels (Fig. 5).

3.7. PCA of physiological responses to Cd stress under different N conditions

To display the general trends of growth and physiological traits, and the patterns of cadmium accumulation and antioxidant defense under contrasting P conditions, PCA was performed based on growth and root traits, anatomical features of stem, Cd accumulations in roots and stems, phosphorus concentrations and N:P ratios, and the levels of antioxidants and phytohormones. PC1 and PC2 accounted for 33.7% and 14.5% of the variation, respectively (Fig. 6). In general, the effects of P conditions were separated by PC1, while the effects of Cd exposure were jointly separated by PC1 and PC2. Dominant variables for PC1 were stem biomass, root N:P

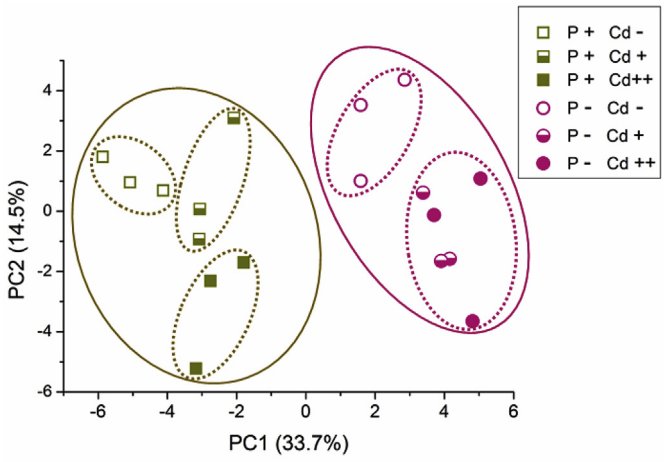

Fig. 6. PCA of growth and physiological traits under combined conditions of phosphorus and cadmium. Growth traits and physiological parameters including Cd concentrations and content, phosphorus concentrations, nitrogen concentration, N:P stoichiometric ratio, the levels of soluble sugars, free proline, chlorophyll, phytohormones and MDA, and the activities of antioxidant enzymes are included in PCA. Square, P-sufficient condition; Circle, P-deficient condition. Open circle or square, 0 mg Cd kg^{-1} sand (CK); Semi-open circle or square, 4 mg Cd kg^{-1} sand (moderate Cd level); Closed circle or square, 20 mg Cd kg^{-1} sand (High Cd level).

ratio, stem P concentration, stem N:P ratio, stem MDA concentration, stem GR activity, root ABA concentration, aboveground biomass, plant R/S, plant biomass (Table S3). The key factors of PC2 were activities of antioxidant enzymes APX and POD in stem, the levels of phytohormones IAA ABA and JA in stems, root biomass and thick root length, Cd concentration and content in stems (Table S3).

4. Discussion

4.1. Growth traits and stem anatomical features were affected by combined conditions of P and Cd

Both phosphorus deficiency and Cd exposure suppressed plant height and biomass production, while the degree of suppression on xylem development was the largest under the combination of Cd exposure and P deficiency. Stem P concentration was not inhibited by Cd exposure, indicating that the absorption of P was not sensitive to Cd stress. Research in *Atriplex atacamensis* showed that arsenate decreased the P concentrations in a less extent than for P starvation (Vromman et al., 2017). Therefore, it can be demonstrated that P absorption by plants are more sensitive to soil P deficiency and less sensitive to heavy metal exposure such as Cd and As.

Phosphorus is essential for nucleic acids, membranes and energy supply during cell division and construction (Thomas et al., 2006; Gan et al., 2016). The present study indicated that both phosphorus deficiency and cadmium exposure inhibit xylem development via reducing cell layers in the xylem. The degree of suppression on xylem development was the largest under the combination of Cd exposure and P deficiency, and the growth suppression was partially alleviated under the combination of Cd exposure and normal P condition. Stem P concentrations (0.3–0.4 mg g^{-1}) was extremely low under P deficiency, and it was approx. four times lower than the mean tissue P concentration (1.45 mg g^{-1}) of China's flora (Wu et al., 2012). In comparison, it was appropriate (1.8–2.0 mg g^{-1}) under normal P condition. Correlation analysis revealed that stem P concentration was positively correlated with xylem thickness and xylem cell layers. It can be concluded that appropriate P status in stem promoted xylem development under high Cd exposure, while the low tissue P concentration increased the degree of growth suppression under Cd exposure due to the reduced cell numbers.

Nitrogen and phosphorus stoichiometry plays vital roles in studying nutrient limitation (Wu et al., 2012). It was proposed that a leaf N:P < 14 indicated N limitation, and leaf N:P > 16 indicated P limitation. The optimal value of N:P ratio in roots or stems is usually lower than that in leaf, because nitrogen demand by the leaf is higher than that by stem due to the photosynthesis function in leaf (Gan et al., 2016; Yan et al., 2016). The concentration of N and P in roots *Populus simonii* Carr under normal nutrient conditions was *approx.* 15.0 mg g^{-1} and 3.0 mg g^{-1} respectively (Gan et al. 2016), implying that a N:P ratio of 5.0 can be suitable for poplar roots or stems. In this study, stem N:P ratios sharply increased from 4.5–4.9 under normal P condition to around 21–23 under P deficiency, indicating a severe P-limitation under deficient-P condition (Wu et al., 2012). Further analysis indicated that N:P ratios in stems had a negative correlation with xylem thickness and xylem cell layers. These results demonstrated that the poor P nutritional status and imbalanced N:P ratio in stems should be responsible for the severe suppression of xylem development under the combined condition of Cd exposure and P deficiency. In contrast, appropriate P status and balanced N:P ratio alleviated growth suppression under high Cd exposure, due to active cell division and construction in stem with adequate supply of nucleic acids, membranes components and energy under normal P condition (Luo et al., 2009; Gan et al., 2016).

4.2. Phosphorus application promoted Cd accumulation in stems via stimulating xylem development

Cd is transported from roots to shoots mainly via the xylem, and thus the xylem structure may influence Cd transport and Cd accumulation in shoots (Qin et al., 2013). Xylem removal dramatically inhibit Cd transportation from roots to above-ground tissues and thus reduce Cd accumulation in shoots (Qin et al., 2013). In the present study, xylem development was markedly promoted by adequate P application as indicated by increased xylem thickness and xylem cell layers. Taken together with the positive correlation between Cd concentration in stems and xylem development, we can deduce that normal P application stimulated cell division and construction of xylem and thus promoted Cd translocation and Cd accumulation in stems. In contrast, the poor P nutritional status and imbalanced N:P ratio under P deficient condition lead to a severe suppression of xylem development under Cd exposure, which inhibited Cd accumulation and Cd bioconcentration in stems.

In addition to affecting Cd translocation, phosphorus can also influence rates of Cd fixation in the cell wall. For example, P deficiency significantly decreased the amount of Cd retained in the root cell wall of *Arabidopsis thaliana* (Zhu et al., 2012), whereas P application increased Cd concentrations in roots of mangrove seedling (Dai et al., 2017b). In the present study, Cd concentration in poplar stems was higher under normal P conditions as compared with P deficiency (Fig. 2). In particularly, Cd distribution in cell walls of collenchyma tissues was far more outstanding under normal P conditions than that in P deficiency, suggesting that exogenous P increased cell wall affinity for Cd and lead to greater Cd accumulation in the cell wall. It was demonstrated that phosphorus might increase Cd accumulation in cell walls via increasing cell wall pectin and hemicellulose 1 concentrations (Dai et al., 2017b). In the present study, the outstanding accumulation of Cd in cell walls of stem collenchyma tissues may be explained as the enhanced capacity of cell walls for Cd binding due to the increasing polysaccharide production with sufficient P application. Moreover, P application may promote the pectin content and PME activity, which can

generate free carboxyl groups available for Cd binding in the cell wall (Dai et al., 2017a). Further analysis revealed that N:P ratios in both stems and roots are negatively correlated with Cd concentration and Cd content in stems, indicating an increasing biological capacity for Cd accumulation and stress tolerance in poplar stems with sufficient P supply and balanced N:P stoichiometry ratio. Additionally, Cd accumulation increased with the increasing intensity of Cd exposure. These results demonstrated that the poplar clone used in this study has strong potential for phytoremediation Cd pollution with sufficient P supply.

4.3. P application and balanced N:P ratio promoted Cd tolerance via enhancing antioxidant defense

Cadmium exposure can lead to concentration-dependent oxidative stress in plants, while plants can combat oxidative stress via inducing the antioxidant systems (He et al., 2015; Shi et al., 2015). Several antioxidant enzymes participate in the scavenging of ROS (reactive oxygen species) such as $O_2^{\bullet-}$ and H_2O_2 (Ma et al., 2013; He et al., 2015). POD play a crucial role in the scavenging of H_2O_2 and hydroxyl radicals (Zhang et al., 2019). In the present study, the activity of POD was increased by high Cd exposure regardless of soil P conditions. Therefore, the role of POD in Cd tolerance was independent of P conditions in the present study. APX and GR participated in the form of ascorbate–GSH cycle that is responsible for scavenging H_2O_2 under stress (He et al., 2015; Ma et al., 2018; Zhang et al., 2019). In the present study, the activity of APX was increased by high Cd exposure when soil P was sufficient, while this induction was not observed under P deficiency. A previous study in *Atriplex atacamensis* indicated that glutathione reductase (EC 1.6.4.2) is affected in some way by P depletion, which will lead to higher sensitivity to As (Vromman et al., 2017). Our results indicated that the extremely low P concentrations (0.3–0.4 mg g^{-1}) and unbalanced N:P ratio under P deficiency can lead to the absent induction of APX and ascorbate–GSH cycle, which should contributed to the increased oxidative stress and lipid peroxidation as indicated by high MDA concentration. Moreover, MDA concentration in a stem is negatively correlated with xylem thickness and cell layers, indicating that increasing MDA level and oxidative stress can lead to greater inhibition on xylem development.

In addition to antioxidant enzymes, phytohormones such as ABA, SA and JA also play crucial roles in combating oxidative stress under heavy metal exposure (He et al., 2011; He et al., 2015; Zhang et al., 2019). Previous studies reported diverse patterns of phytohormone inducement under heavy metal exposure, which may vary across plant species and/or environmental factors (Sofo et al., 2013; Shi et al., 2015; Wang et al., 2019). Under normal P condition in the present study, GA_3 and SA were increased by high Cd exposure. However, all of the tested phytohormones (ABA, JA, GA_3 and SA) were decreased by Cd exposure under P deficiency. It was reported that although of a variety of antioxidants can combat Cd

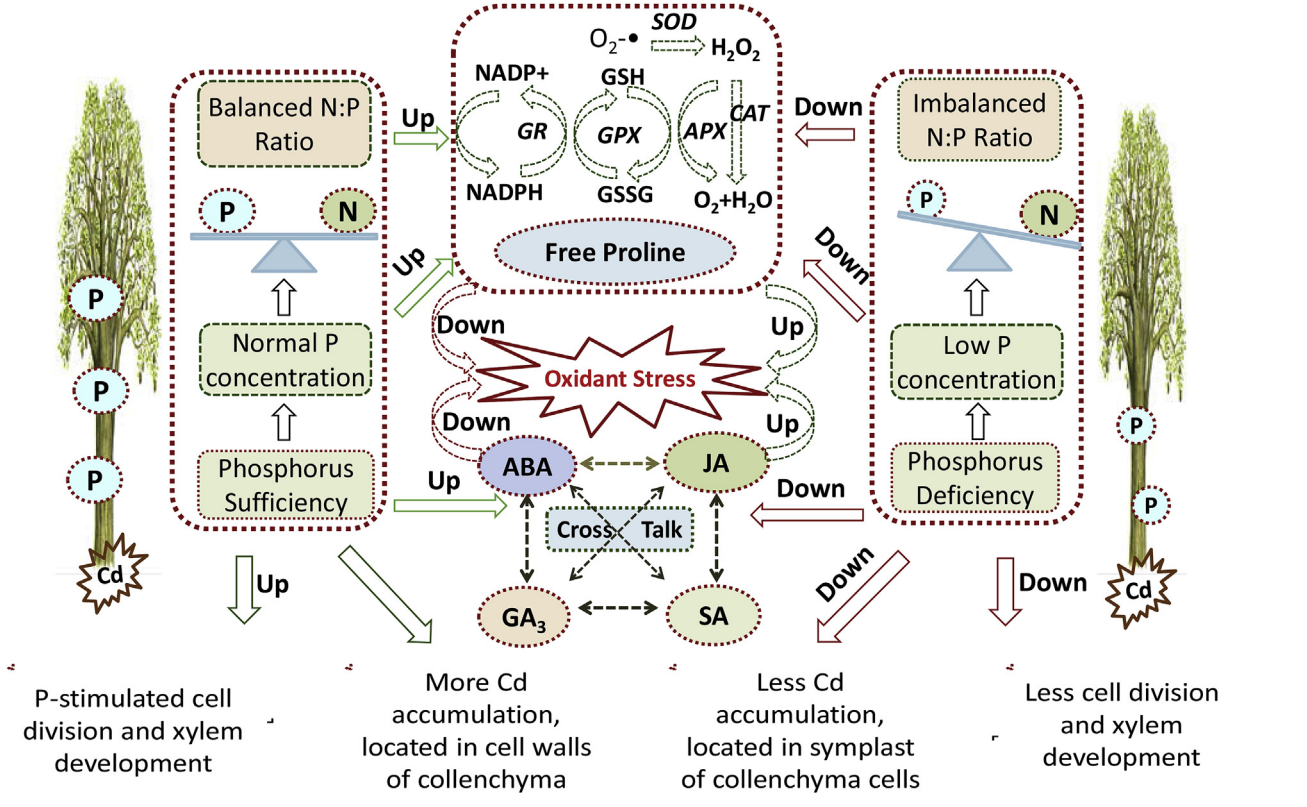

Fig. 7. Schematic model of mechanisms underlying the influence of phosphorus status and N:P stoichiometric ratio on cadmium accumulation and stress tolerance in *Populus* stems. Under P-sufficient condition, cell division and xylem construction were up-regulated by proper tissue P concentration and balanced N:P ratio, which enhanced Cd translocation and increased Cd accumulation in stems. In contrast, the extremely low tissue P concentration and imbalanced N:P ratio under P deficiency reduced cell division and inhibited xylem development, and thus decreased Cd accumulation in stems. Phosphorus addition also increased cell wall affinity for Cd and thus enhanced Cd accumulation in stem cell wall, presumably due to the active polysaccharide production after P application. The activity of APX was down-regulated by low P concentrations and imbalanced N:P ratio under P deficiency, which increased the oxidative stress in stem. A negative interaction between phytohormones and antioxidant enzymes was detected in poplar stems under combined conditions of P deficiency and Cd exposure. In contrast, the positive interactions between phytohormones and antioxidants contributed to the favoring antioxidant defense under normal P conditions. In conclusions, proper P addition and balanced N:P ratio can enhance phytoextraction of Cd from Cd-contaminated sand via enhancing secondary xylem development, up-regulating antioxidant defense and promoting cadmium accumulation in *Populus* stems.

stress, severe Cd stress may also conversely disturb or interfere with the biosynthesis of these antioxidants including phytohormone, GSH and PC (Zhu et al., 2012; Wang et al., 2019). Our results indicated that the inducement of phytohormones upon Cd exposure were highly related to P status. When soil P was deficient, the biosynthesis of various phytohormones were inhibited by high Cd exposure, presumably due to the increasing oxidative stress as a result of the absent induction of antioxidant enzymes such as APX and GR under P deficiency. The patterns revealed by the present study can be considered as a negative "cross-talk" between phytohormones and antioxidant enzymes when soil P deficiency and Cd exposure synchronously occurs.

Under normal P condition, the concentrations of some specific phytohormones (SA and GA_3) were promoted by high Cd exposure. It was reported that SA can stimulate N and S metabolism, and thus increase the levels of APX and GR, which can contribute to stress tolerance and growth inhibition under stress (Nazar et al., 2011; Wang et al., 2019). Interestingly, a sharp inducement of antioxidase activity (APX, GR and POD) upon Cd exposure was also observed under normal P conditions of the present study. These findings implying the interactions between phytohormones and antioxidants can play crucial roles in the process of antioxidant defense under Cd exposure (He et al., 2015), which at least partially dependent on normal P nutritional conditions and balanced N:P ratio.

5. Conclusions

The mechanisms underlying the influence of phosphorus status and N:P stoichiometric ratio on cadmium accumulation and stress tolerance in *Populus* stems were explored (Fig. 7). Under P-sufficient condition, sufficient tissue P concentration and balanced N:P ratio enhanced cell division and xylem construction and thus promoted Cd translocation and increased Cd accumulation in stems. In contrast, the extremely low tissue P concentration and imbalanced N:P ratio under P deficiency down-regulated cell divisions and reduced cell numbers and cell layers, which may inhibit Cd accumulation in stems. Phosphorus addition increased cell wall affinity for Cd and enhanced Cd accumulation in stem cell wall, presumably due to active polysaccharide production after P application. The low P concentrations and imbalanced N:P ratio under P deficiency inhibited APX production and ascorbate–GSH cycle, which lead to increasing oxidative stress in stem. A negative interaction between phytohormones and antioxidant enzymes was detected in poplar stems when soil P deficiency and Cd exposure synchronously occur. In contrast, the positive interactions between phytohormones and antioxidants was crucial for antioxidant defense under normal P conditions. In conclusions, appropriate P addition and balanced N:P ratio can enhance phytoextraction of Cd from Cd-contaminated soil via enhancing secondary xylem development, up-regulating antioxidant defense and promoting cell wall affinity for Cd in *Populus* stems.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.125154.

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