

Boron Mitigates Cadmium Toxicity by Reducing Cadmium Accumulation, Enhancing Cell Wall Immobilization and Regulating gene expression in Malus Rootstock

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Abstract

To investigate the mitigating role and underlying mechanisms of exogenous boron (B) in cadmium (Cd)-stressed woody fruit trees, a hydroponic study was conducted using *Malus hupehensis* Rehd. seedlings treated with different B concentrations (0, 12.5, 50, and 150 μM H_3BO_3). Cd stress significantly inhibited plant growth, reduced photosynthetic parameters, pigment content, biomass, and root activity, but induced reactive oxygen species (ROS) accumulation and impaired the antioxidant defense system. In contrast, the 50 μM B treatment (B2) effectively alleviated Cd toxicity. This treatment significantly decreased Cd accumulation, bioconcentration factor, and translocation factor across tissues. The B2 treatment enhanced Cd immobilization in root cell walls by increasing pectin content and pectin methylesterase activity. Additionally, it shifted Cd chemical forms toward lower-toxicity forms—increasing pectin- and protein-bound, phosphate-bound, and oxalate-bound Cd, while reducing inorganic and water-soluble Cd fractions. The B2 treatment further activated the antioxidant system, elevating the activities of superoxide dismutase and peroxidase, and increasing non-enzymatic antioxidant levels (free proline and ascorbic acid), thereby reducing ROS and malondialdehyde accumulation. The B2 treatment also downregulated key genes including *ZIP6* and *IRT1* involved in Cd uptake. In conclusion, an optimal B concentration of 50 μM alleviates Cd stress in *Malus hupehensis* Rehd. by regulating Cd uptake and translocation, enhancing cell wall fixation, altering Cd chemical forms, activating antioxidant defenses, and regulating stress-related gene expression.

1 Introduction

Heavy metal pollution is a severe environmental challenge for the survival of animals and plants, among which cadmium (Cd) poses the most serious threat to plant growth and human health (Liu et al., 2024). In China, about 7% of arable land has been polluted by Cd (MEP 2014). Recently, inappropriate management and fertilization have led to Cd accumulation in orchards. For example, a study conducted in orchards across Shaanxi Province revealed that 10.0% of soil samples contained detectable levels of Cd, whereas 52.5% of fruit samples exceeded the national maximum allowable limit for Cd (Guo et al., 2016). Cd is highly biotoxic and difficult to degrade, and can be easily absorbed and accumulated by plants. Cd accumulated in fruit tree can damage photosynthetic organs, decrease photosynthetic pigments, inhibit photosynthesis, induce oxidative stress, damage cell membrane structure and function, inactivate enzymes and proteins, and finally restrain plant growth and lower crop yield (Mittler, 2017, Ullah et al., 2021, Huang et al., 2021a, Zhuang et al., 2025). Furthermore, Cd can enter the human body through the food chain and posing a significant threat to human health (Zhou et al., 2023). Therefore, it is necessary to find some efficient, environmentally friendly and cost-effective strategies to reduce Cd accumulation and improve Cd resistance in woody fruit trees.

Boron (B) is a trace element required for many physiological and metabolic functions in plants. Previous studies have demonstrated that B is involved in cell division and elongation, nucleic acid metabolism, biofilm structure and function maintenance, enzyme activation, nitrogen and carbohydrate metabolism, sugar transport (Shireen et al., 2018, Matthes et al., 2020). Furthermore, B is proposed in many plants to

affect Cd accumulation and resistance. For example, exogenous B can significantly inhibit the absorption and transport of Cd in wheat, especially in the seedling stage and the growth stage (Qin et al., 2022). Similarly, B addition significantly decreased Cd translocation from root to shoot in rice (Riaz et al., 2021a). However, a narrow range exists between B deficiency and toxicity. For example, Liu et al. (2018) found appropriate B supply promote plant growth, but over 50 μM of B on plant exhibited toxicity symptom. Therefore, suitable concentration of B must be screened before applying B to reduce Cd toxicity. Recent studies have shown that appropriate B application alleviated Cd toxicity by activating the defense mechanisms in rape (Qin et al., 2022), wheat (Qin et al., 2020), rice (Huang et al., 2021c). However, no information is available on whether appropriate B application could alleviate Cd stress in apples.

Exogenous B enhances Cd detoxification in herbaceous plants via several physiological mechanisms (Huang et al., 2023). First, B can affect the cell wall's ability to fix Cd by regulating the components of the plant cell wall (such as cellulose, hemicellulose, lignin and pectin) and their structure. The active groups, such as carboxyl (COO^-), hydroxyl ($-\text{OH}$), and thiol ($-\text{SH}$) groups in these cell wall components are the key sites for chelating Cd (Kushwaha et al., 2015, Wan et al., 2021). When B is deficient or excessive, it may change the physicochemical properties of the cell wall, thereby weakening its blocking effect on Cd through ion binding and fixation mechanisms (Krzesłowska, 2011). Under Cd stress, the application of B increased the pectin content of cell wall, improved the pectin methylesterase activity, reduced the degree of pectin methyl ester, and then enhanced the chelation of Cd by root cell wall, thus reducing the level of Cd entering the organelles in *Brassica napus* (Wu et al., 2020a). Wu et al. (2020c) also found that exogenous addition of $0.25 \text{ mg} \cdot \text{L}^{-1}$ B increased the contents of pectin and cellulose in young stems of *brassica napus*, promoted the fixation of Cd in cell wall, enhanced the resistance of young stems to Cd, and reduced the toxicity of Cd. Second, B affects the vacuolar compartmentalization capacity for heavy metals, with vacuoles playing a critical role in the immobilization, inactivation and detoxification of heavy metals (Sharma et al., 2017). B regulates the activity of vacuolar membrane transport proteins, which utilize proton gradients or ATP hydrolysis to compartmentalize Cd into vacuoles (Sharma et al., 2016, Park et al., 2012). In *Arabidopsis*, B deficiency disrupts vacuolar membrane integrity, whereas B supplementation restores vacuolar membrane function, ensuring efficient Cd compartmentation (Miwa and Fujiwara, 2010). Additionally, B influences the chelation of Cd by modulating the synthesis of phytochelatins (PCs); deficiency B reduces Cd-PC complex formation and vacuolar sequestration efficiency, as observed in rice (Luo et al., 2018). Third, B affects plant Cd tolerance by regulating cell ROS homeostasis (Yin et al., 2021). The appropriate concentration of B improves Cd resistance by regulating antioxidant enzymes and non-enzymatic antioxidants, eliminating excessive accumulated ROS in chili peppers and rice (Riaz et al., 2021b, Huang et al., 2021b). Fourth, B affects the expression of genes involved in Cd absorption and detoxification processes. When B is deficient, the expressions of *iron-regulated transporter 1 (IRT1)* and *Zrt-/Irt-like protein 6 (ZIP6)* are upregulated, promoting Cd to enter root cells; However, an appropriate supply of B can inhibit these transporters and reduce Cd accumulation (Xin et al., 2023). B also promotes the transport of Cd to vacuoles by up-regulating the expression of vacuole dissociation-related transport proteins such as *metal tolerance protein 1 (MTP1)* and *magnesium*

proton exchanger protein (MHX) (Quiles-Pando et al., 2013, Zhuang et al., 2025). Up to now, there are currently no studies or possible mechanisms on B enhancing the detoxification effect of Cd in woody fruit crops especially in apple plants.

Soil Cd pollution in orchard restricts the healthy and sustainable development of fruit industry. Appropriate B application could be a viable candidate in the mitigation of Cd toxicity. However, the alleviating effect of B on Cd stress in woody fruit trees, especially apple plants, and its mechanism have not been reported. This experiment employed *Malus hupehensis* Rehd., a commonly used rootstock known from previous study to exhibit poor Cd tolerance (Zhou et al., 2017). To examine whether appropriate B would alleviate Cd toxicity and the underlying physiological mechanism of B affecting Cd uptake, accumulation and detoxification in *M. hupehensis*, we exposed it to either 0 or 50 μM Cd together with 0, 12.5, 50 or 150 μM B for 30 days. We hypothesized that (i) appropriate B would reduce Cd migration and accumulation, while enhancing Cd tolerance in apple plants, and (ii) B would alleviate Cd toxicity in apple plants by regulating the physiological basis and the expression of genes related to Cd absorption and vacuolar compartmentalization. To test these hypotheses, we measured plant growth parameters, antioxidant defense system, and Cd adsorption of the CW, and to study the variation in the CW functional groups by FTIR analysis. The present results will enhance the understating the mechanisms of B-induced alleviation of Cd toxicity in *Malus* and provide a foundation for the developing of reliable methods to reduce Cd accumulation and enhance Cd tolerance in apple and other fruit trees.

2 Materials and methods

2.1 Plant material cultivation and treatment

The experimental materials were derived from seeds of *Malus hupehensis* Rehd..

The seeds of *Malus hupehensis* Rehd. were collected at the Fruit Tree Scientific Research Base of Shenyang Agricultural University, located in Shenyang, Liaoning Province, China. The seedlings of *Malus hupehensis* Rehd. were stratified in sand at 0–4°C for 40 d, After germination, the seeds were cultivated for 40 days in greenhouse under natural light (day/night temperature: 26/18 °C, 50–60% RH) using nursery plates with seedling matrices at Shenyang Agricultural University, Shenyang, China (41°49'N, 123°34'E). One month later, plants with similar height were transferred to plastic pot (20 cm × 20 cm × 18 cm) containing 4 kg clean sand. The plant in each pot was poured 100 ml distilled water every morning, and irrigated every other day with 50 mL 1/4 Hoagland nutrient solution. When the seedlings grew to 20 cm high, 96 seedlings with the same growth were selected and transferred to a hydroponic tank for further cultivation. They were then divided into 8 groups (12 plants per group) on average. Each tank is equipped with a 7 W ventilation pump, and set B0 (0 μM H_3BO_3), B1 (12.5 μM H_3BO_3), B2 (50 μM H_3BO_3), B3 (150 μM H_3BO_3) by changing the amount of H_3BO_3 in the nutrient solution, then the seedlings were treated with 0 or 50 μM CdCl_2 , the nutrient solution was changed every 2 days, and the test materials were harvested after 30 days of treatment.

When harvesting test materials, the roots were rinsed with 0.05 mM CaCl₂ and then rinsed 3 times with distilled water to remove excess Cd from the root surface. The plants were divided into three parts: roots, stems and leaves, and the fresh weight was recorded, then frozen in liquid nitrogen with a ball mill (MM400; Retsch GmbH, Haan, Germany) fully ground and stored in a -80 °C refrigerator. Equal amounts of fine powder from the same tissue of four plants with the same treatment were pooled. Therefore, each treatment had three replicates for subsequent determination of physiological and biochemical indicators.

2.2 Determination of photosynthetic and fluorescence parameters, and growth characteristics

Before harvest, the net photosynthetic rate (P_n), stomatal conductance (G_s) and transpiration rate (T_r) of mature leaves were measured by CIRAS-2 portable photosynthesizing apparatus (PP Systems, USA).

The concentrations of chlorophylls and carotenoids in the leaves were analyzed as previously described (Alan, 1994). And placed in darkness to the sample after completely turning white, the pigment content was determined by ultraviolet spectrophotometer (UV-3802, Unico Instruments Co. Ltd, Shanghai, China) at 663, 646 and 470 nm, respectively (Wellburn 1994).

The fresh samples (100 mg) from each tissue per plant were put into a centrifuge tube with constant weight and dried at 60 °C for 72 h to determine the fresh-to-dry mass ratio, then the biomass of each tissue was calculated.

The root configuration parameters were analyzed using a WinRHIZO Root Analyzer System (WinRHIZO 2012b, Regent Instruments Canada INC., Montreal, Canada). TTC method was used to determine the root activity at the root tips of plants, referring to the method of (Richter et al., 2007).

2.3 Determination of Cd concentration, Cd bio-concentration factor (BCF) and Cd translocation factor (Tf)

Fresh powders (500 mg) of each tissue sample were add 7 ml HNO₃ and 1 ml HClO₄, after standing overnight, it was placed on a constant-temperature digestion plate at 170°C for digestion according to the method of Zhou et al. (2016). After digestion, the concentration of Cd was determined by flame atomic absorption spectrophotometer (Hitachi 180 – 80, Hitachi, Tokyo, Japan).

The Cd bio-concentration factor (BCF) and Cd translocation factor (Tf) were calculated Using following equations, respectively:

$$BCF = C_{\text{root/stem/leaf}} \times C_s$$

$$Tf = C_{\text{stem/leaf}} / C_{\text{root}}$$

$C_{\text{root/stem/leaf}}$ represents the Cd concentration in roots, stems, and leaves, and C_s represents the Cd content in the solution.

2.4 Analysis of subcellular distribution and chemical forms of Cd

The subcellular components of the plants were separated according to (Yan et al., 2022). After the separated components were slightly dried and dissolved on a cooking plate (HNO_3 : HClO_4 =7:1), the content of Cd was determined by flame atomic absorption spectrophotometer (Hitachi 180 – 80, Hitachi, Tokyo Japan).

Determination of Cd chemical forms was carried out according to Wu et al. (2005). The obtained supernatant was dried at 70°C and dissolved with HNO_3 and HClO_4 (7:1), the content of Cd in each component was determined by flame atomic absorption spectrophotometer (Hitachi 180 – 80, Hitachi, Tokyo, Japan).

2.5 Fourier transform infrared (FTIR) spectroscopy analysis

Using the an FTIR spectroscope (VERTEX70; (Bruker Crop, MA, USA), weigh 200 mg of potassium bromide and 2 mg of root cell walls into a mortar, grind evenly and then prepare slides. Determine its infrared spectrum under the same conditions. The spectral resolution is 4 cm^{-1} and the scanning range is $4000\text{--}400\text{ cm}^{-1}$.

2.6 Cell wall analysis

2.6.1 Determination of Cd content in each component of cell wall

Root cell wall samples were collected as previously described (Kang et al., 2015) with some modifications. Briefly, 1.0 g frozen root samples were homogenized in pre-cooled 75% (v/v) ethanol for 20 min and centrifuged at $5000\times g$ (4 °C for 10 min). These steps were repeated three times. The pellets were washed with ice-cold acetone, 1:1 (v/v) methanol: chloroform mixture, and methanol, sequentially (20 min per step). The supernatant was discarded, and the final pellet comprised the crude cell walls. After freeze-drying, the cell walls were stored at 4 °C for further analysis. Two milligrams root cell wall isolate was mixed with 200 mg KBr and pulverized in an agate mortar. The samples were measured using an FTIR spectroscope (VERTEX 70; Bruker Corp., Bill-erica, MA, USA) within the $4000\text{--}400\text{ cm}^{-1}$ scanning range and at 4 cm^{-1} resolution.

After the separated components were slightly dried and dissolved on a cooking plate (HNO_3 : HClO_4 =7:1), the content of Cd was determined by flame atomic absorption spectrophotometer.

2.6.2 Cell wall component content determination

The concentrations of pectin were determined by m-hydroxybiphenyl method according to the method of Chudzik et al. (2018).

The content of HC1 HC2 and cellulose was determined according to Zhu et al. (2013), components referred to previous descriptions with some modifications.

2.6.3 Assay of cell wall related enzyme activity

CW enzymes were extracted according to the method of Wu et al. (2020b). The 0.02 g sample was weighed and 2 ml 1 mol/L NaCl (containing 20 mmol/L Tris-HCl, pH 4.8) was used as the extraction solution, centrifuged for 15 min for 10000 r (4°C), and the separated supernatant was used as the crude enzyme extraction solution. After that, the activities of β -glucosidase and carboxymethyl cellulase were measured at 540 nm. The activity of pectin methyl esterase was measured at 450 nm.

2.7 Determination of O_2^- , H_2O_2 , MDA content

The concentrations of O_2^- and H_2O_2 in the root, stem and leaf tissues were determined spectrophotometrically at 530 and 410 nm, respectively, as suggested by Zhang et al. (2010) and He et al. (2011). The concentrations of MDA in plant tissues were determined with a spectrophotometer as described by Zhou et al. (2016).

2.8 Analysis of non-enzymatic antioxidants and enzymatic antioxidant activity

The content of free proline was determined according to Tamás et al. (2008). Determination of soluble phenol content by Folin–Ciocalteus reagent according to the method of Luo et al. (2008). The contents of AsA and total thiols (T-SH) were determined according to the methods of He et al. (2013).

The determination of soluble protein to calculate antioxidant enzyme activity refer to Luo et al. (2008). The enzyme activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) were measured according to the method of Wang et al. (2013).

2.9 Gene expression analysis

The RNA from the root was extracted by CTAB method (Lay-Yee et al., 1990). cDNA was obtained from the extracted RNA by reverse transcription kit (RR047A, Takara, China), and the cDNA was diluted by 10 times for real-time quantitative fluorescent PCR (Li et al., 2022). A 10 μ L reaction system was established, including 5 μ L of 2 $\times$ SYBR Green Premix EX Taq II (DRR820A, Takara, Dalian, China), 1 μ L of diluted cDNA and 1 μ L of 10 mM upstream and downstream primes. The procedure is as follows: 95°C predenaturation 10 min, 95°C 15 s, 60°C 60 s, return to the second step of the program, a total of 40 cycles; store at 72°C for 5 min and at 4°C. β -actin was used as the internal reference for fluorescence quantification. In order to ensure specificity, specific primers were designed for each gene (Table S1). The relative expression of genes was calculated by $2^{-\Delta\Delta CT}$.

2.10. Data statistics and analysis

Statgraphics (STN, St.Louis, MO, USA) software was used for statistical analysis of data. After all data were checked for normal distribution, two-way analyses of variance (ANOVAS) was performed, and H_3BO_3 addition (B) and Cd treatment (Cd) were the two main factors. The difference was significant when P -values ≤ 0.05 by F test of ANOVA. The figures were drew using Origin 2021. Heat map rendering using OmicStudio (<https://www.omicstudio.cn/>).

3 Results

3.1 Growth characteristics

In the absence of Cd, Pn, Tr, Gs and photosynthetic pigments of *M. hupehensis* increased first and then decreased with the increases of B concentration (Fig. 1a-c and Table S2). Cd stress significantly decreased Pn, Gs and photosynthetic pigments, regardless of B addition, with the exception of Gs, Chl(a) and Car under B2 condition. The detrimental effects of Cd on those above parameters were alleviated after the application of 50 μ M B but aggravated with the supplement of 150 μ M B (Fig. 1a-c and Table S2).

Consistent with Cd-induced photosynthetic inhibition, Cd exposure significantly reduced the root, stem and leaf biomass of seedlings, except for root biomass under B0 condition and stem biomass under B2 and B3 conditions (Fig. 1d-f). Under Cd exposure treatment, tissue biomass initially increased but then declined with rising B concentrations, reaching its peak in the B2 + Cd treatment (Fig. 1d-f).

In the absence of Cd, with the increase of B concentration, the total root length, total root volume, total root surface and root activity of plants first increased and then decreased (Table S3). Under Cd stress, B application significantly increased root surface area, total root volume and root vitality compared to the B0 + Cd treatment, with the most pronounced effects observed under B2 + Cd treatment (Table S3).

3.2 Cd concentration, BCF and T_f

Under Cd treatment, B application significantly reduced Cd concentration in the roots, stems and leaves compared to the B-free treatment, with B2 + Cd treatment displaying the most pronounced effects (Fig. 2a-c). Specifically, the B2 + Cd treatment reduced Cd concentration in the roots, stems and leaves by 25.23%, 32.60% and 44.72%, respectively, relative to the B0 + Cd treatment (Fig. 2a-c). The BCF in the roots and aerial organ and T_f of *M. hupehensis* showed an initial decrease followed by an increase with rising concentration of B, reaching its minimum under the B2 + Cd treatment (Fig. 2d, e). Compared to the B0 + Cd treatment, the B2 + Cd treatment reduced the BCF by 38.52% in roots and 25.23% in aerial parts, and decreased the T_f by 21.25% (Fig. 2d, e).

3.3 Different forms and subcellular distribution of Cd

In both roots and leaves of *M. hupehensis*, Cd proportion in pectates and protein integrated forms (extracted by 1 M NaCl) were highest, followed by water soluble fraction (extracted by deionized water and corresponded to the most toxic form of metals) (Fig. 3a, b). Under Cd stress, the proportion of Cd in inorganic (extracted by 80% ethanol) and water-soluble Cd in roots decreased first and then increased with the increase of B concentration, with B2 + Cd exhibiting the lowest value (Fig. 3a). On the contrary, the proportion of pectin and protein-bound Cd and undissolved Cd phosphate form (extracted by 2% HAC) in the roots showed an initial increase followed by a decrease with rising concentration of B, with B2 + Cd treatment showing the highest value (Fig. 3a). Compared with the B0 + Cd treatment, the addition of different concentrations of exogenous B reduced the proportion of Cd in the oxalate state (Fig. 3a).

In the leaves, B2 + Cd treatment resulted in the lowest levels of inorganic and water-soluble Cd compared to B-free and other B treatments (Fig. 3b). There was no significant difference in the proportion of pectates and protein-bound Cd among different B treatments (Fig. 3b). Compared with B0 + Cd treatment, both B2 + Cd and B3 + Cd treatments increased the proportion of insoluble phosphate Cd and oxalate Cd, with the most pronounced increase observed under B2 + Cd (Fig. 3b).

In the roots, the addition of various B concentrations increased the proportion of Cd in the cell wall compared with the B0 + Cd treatment, with the B2 + Cd treatment being the highest (Fig. 3c). On the contrary, B2 + Cd treatment significantly decreased the proportions of Cd in the plastid, mitochondria and vacuole of roots compared to B0 + Cd (Fig. 3c).

In the leaves, the proportion of Cd in the cell wall were increased by 19.06%, 31.47% and 24.9%, respectively, in the B1 + Cd, B2 + Cd and B3 + Cd treatment than those in the B0 + Cd treatment (Fig. 3d). Compared with B0 + Cd, the proportion of Cd in plastids and nucleus was only decreased in the B1 + Cd treatment. The addition of all B concentrations decreased the proportion of Cd in ribosomes relative to the B0 + Cd treatment, but no significant difference was observed among the three treatments (Fig. 3d). Cd proportion in the vacuoles were relative lower in the B2 + Cd and B3 + Cd treatments than those in the B0 + Cd and B1 + Cd treatments (Fig. 3d).

3.4 Analysis of Cd content in each component of root cell wall, FTIR, root cell wall components and cell wall metabolic enzyme activities

Compared with B0 + Cd treatment, addition of B significantly increased Cd content in pectin, with B2 + Cd showing the most pronounced effects. Besides, B2 + Cd treatments significantly increased Cd content in HC1 and cellulose, respectively, relative to B0 + Cd treatment (Fig. 4a). Surprisingly, addition of B reduced the Cd content in HC2, except for B3 + Cd treatment in comparison with no B treatment (Fig. 4a). In general, the proportion of Cd in pectin were highest, followed by cellulose, and Cd proportion in HC1 and HC2 were relative low in the roots of *M. hupehensis* (Fig. 4b). Consistent with the results of Cd content in various cell wall components, compared with the B-free treatment, the three B concentration treatments significantly increased the proportion of Cd in pectin and cellulose, except for Cd proportion in pectin under B3 + Cd treatment (Fig. 4b).

FTIR analysis was conducted to characterize the functional groups in the root cell of *M. hupehensis* subjected to B and Cd treatments (Fig. 4c). A peak at $\sim 3413\text{ cm}^{-1}$ represented the O-H or N-H stretching vibration of fatty acids, proteins, pectin, and hemicellulose. A peak at $\sim 2924\text{ cm}^{-1}$ represented the C-H stretching vibration of a lipid carbon chain ($-\text{CH}_3$, $=\text{CH}_2$, $=\text{CH}-$) derived mainly from hydrophilic lipid molecules. A peak at $\sim 1738\text{ cm}^{-1}$ represented the vibrations of $-\text{C}=\text{O}$ in pectin. A peak at 1639 cm^{-1} represented the vibrations of C-N in proteins. Numerous absorption peaks were detected in the $1517 - 1253\text{ cm}^{-1}$ range and represented the symmetric bending vibration of N-H in proteins, while the unsymmetrical stretching vibration of carboxylate COO^- , the stretching vibration of sulfate $-\text{C}-\text{O}-\text{S}$, the vibration of phosphate in $\text{C}-\text{O}-\text{P}$, and the stretching vibration of carboxyl $-\text{C}-\text{O}$. A peak at 1154 cm^{-1} represented the polysaccharide ring structure of C-C or C-O. A peak at 1041 cm^{-1} represented the -CH bending or -C-C, -C-O stretching vibration peak insoluble sugars, cellulose sugar chains, and hemicellulose. Regardless of B and Cd stress, the spectral peak shape of plant root cell walls did not change significantly. Only the characteristic absorption peaks occurred to different degrees. Cd stress resulted in a significant increase in each absorption peak of the root cell wall, regardless of the concentration of B. Under Cd stress, the intensity of each absorption peak increased significantly under B2 + Cd treatment except for 1050 cm^{-1} (Fig. 4c).

With the increase of B concentration, the pectin content first increased and then decreased, reaching its peak after application of $50\text{ }\mu\text{M}$ B, regardless of Cd treatment (Fig. 4d). In general, Cd stress increased the content of pectin in all treatment with the exception of $12.50\text{ }\mu\text{M}$ B addition, and the highest value was observed in the B2 + Cd treatment. After Cd exposure, the content of HC1 and HC2 were significantly increased in all treatments except for HC1 under B free and $150\text{ }\mu\text{M}$ B addition treatments (Fig. 4d). Surprisingly, Cd stress had no significant effects on the cellulose content of roots after addition of B (Fig. 4d).

In the absence of Cd, β -glucosaccharase, methylcellulase and pectin methylase activities were significantly increased after B addition except for the first two enzymes under B1 treatment (Fig. S1). Under Cd stress, the activities of β -glucosidase and pectin methylase were significantly increased then decreased with the increase of B addition concentrations, with B2 + Cd treatment showing the highest values (Fig. S1). There was no significant difference in methylcellulase activity among different treatments under Cd stress (Fig. S1).

3.5 Reactive oxygen species and antioxidants in seedlings

Cd stress significantly increased the contents of $\text{O}_2^{\cdot-}$, H_2O_2 and MDA in the roots and leaves of *M. hupehensis* (Fig. 5). However, under Cd stress, the contents of $\text{O}_2^{\cdot-}$, H_2O_2 and MDA in the roots and leaves of *M. hupehensis* were significantly lower in the B2 + Cd treatment than the other three treatments (Fig. 5). In contrast, $\text{O}_2^{\cdot-}$, H_2O_2 and MDA level in the B0 + Cd treatment were generally higher than in other treatments.

In general, Cd stress led to significant decrease in the free proline contents in roots and leaves under no B and low-concentration B treatments (Fig. S2). However, under Cd exposure conditions, there was a significant increase in root proline contents after application of 50 μ M B (Fig. S2). Cd stress significantly decreased T-SH in the roots, but increased T-SH in the leaves of all plants, irrespective of B addition. The T-SH content was highest after 50 μ M B addition, regardless of Cd treatment. Cd stress significantly increased in root soluble phenolics only under the B2 + Cd treatment, and leaf soluble phenolics only under the B3 + Cd treatment, compared to their respective controls. Under Cd stress, compared with B0 + Cd, B3 + Cd treatment significantly increased the content of ASC in roots, and all three B addition treatments significantly elevated ASC content in leaves (Fig. S2).

Cd stress had no significant effect on the SOD activity in roots and leaves, except for SOD activity in leaves under B3 + Cd treatment (Fig. S3). Under Cd stress, the SOD activity in the roots and leaves were always higher in B1 + Cd and B2 + Cd treatments than the other two treatments (Fig. S3). After Cd stress, the root and leaf POD activity of roots and leaves treated with B2 + Cd was significantly increased (Fig. S3). Under Cd stress, compared with B0 + Cd, B1 + Cd treatment significantly increased CAT activity in the root and no significant differences were observed in the CAT activity among different treatments. Except for the B0 + Cd treatment, Cd stress significantly increased the root APX activity. However, Cd stress reduced the APX activity of the leaves, irrespective of B addition (Fig. S3).

3.6 Gene expression analysis

In the absence of Cd stress, the expression of *ZIP6* was significantly down-regulated in all B addition treatments compared with B0 (Fig. 6a). Under Cd stress, *ZIP6* expression were downregulated by 6.14–fold and 1.72–fold under B2 + Cd and B3 + Cd treatments, respectively, compared with the B0 + Cd treatment (Fig. 6a). Similarly, under Cd stress, the expression of *IRT1* in B2 + Cd treatment was also significantly lower than that in the other three treatments (Fig. 6b). Compared to the Cd-free controls, Cd exposure increased MTP1 transcript levels in the roots under all B treatments except for the B0 condition. Irrespective of Cd treatment, MTP1 expression remained lower under the 50 μ M B treatment (Fig. 6c). Similarly, compared to the Cd-free controls, Cd exposure generally up-regulated MHX transcript levels. In parallel with the trend observed for MTP1, MHX expression was also consistently lower under 50 μ M B treatment regardless of Cd exposure (Fig. 6d).

4 Discussion

4.1 Appropriate B reduced Cd accumulation and enhanced Cd tolerance in apple rootstock

The toxic effects of Cd stress on plants are primarily characterized by a significant inhibition of photosynthetic systems and root growth (Wang and Wang, 2006). The results of this study clearly showed that 50 μ M CdCl₂ treatment (B0 + Cd) led to a significant decrease in the contents of chlorophyll a, b and carotenoids and photosynthetic system parameters in *Malus hupehensis* Rehd. leaves (Fig. 1

and Table S2), and at the same time, the total root length, root volume and root vitality of the root system were also severely damaged (Table S3). These results indicated that Cd stress severely disrupted the photosynthetic structure and root development of *Malus hupehensis* Rehd., which is consistent with the results of previous study (Liu et al., 2025).

Exogenous application of B can effectively alleviate the toxicity of Cd, but this alleviating effect has a concentration effect (Brown and Hu, 1997; Lu et al., 2019). Under Cd stress, the application of 50 μ M B exhibited the most pronounced alleviative effect. It significantly restored parameters associated with the photosynthetic system, including the contents of chlorophyll a, chlorophyll b, and carotenoids (Table S2), and effectively enhanced total root length, root volume, and root vitality (Table S3). This phenomenon can likely be attributed to the fact that the application of 50 μ M B significantly reduced Cd accumulation in the roots and its translocation to the aerial parts (Fig. 2). However, compared with the 50 μ M B treatment, a lower B concentration (12.5 μ mol) exhibited a reduced alleviative effect on Cd stress, whereas a higher B concentration (150 μ mol) even exacerbated Cd toxicity. These results suggest that 50 μ M boron represents an appropriate concentration for effectively mitigating Cd stress in *Malus hupehensis* Rehd. This optimal B level may activate specific plant defense mechanisms, thereby reducing Cd mobility and enhancing plant tolerance.

4.2 Appropriate B supply enhanced Cd tolerance by modulating Cd chemical forms and cell wall binding capacity

The toxicity degree and mobility of Cd in plants are dependent on its chemical forms inside cells (Su et al., 2014). Overall, inorganic and organic Cd (extracted by 80% ethanol and deionized water) have stronger migration ability and greater toxicity to plant cells than other Cd chemical forms (Wu et al., 2005; Wang et al., 2008). In the present study, the appropriate concentration of B treatment (50 μ M) significantly reduced the proportion of highly mobile and toxic inorganic and water-soluble Cd in roots and leaves, while increasing the proportion of less mobile and less toxic pectin protein-bound and insoluble phosphate-bound Cd (Fig. 3a, 3b). This shift indicates that 50 μ M B alleviate Cd toxicity by reducing the proportion of highly mobile and phytotoxic Cd species and promoting their conversion into less bioavailable immobilized forms. The increased proportion of pectate/protein-integrated Cd further indicates that pectin plays a critical role in Cd immobilization, which is consistent with findings in Cd-stressed rapeseed plants (Wang et al., 2015).

At the subcellular level, root cell walls serve as the primary barrier preventing Cd from entering the cytoplasm. In this study, under Cd stress, treatment with 50 μ M B significantly increased the content and distribution ratio of Cd in the cell wall components of roots and leaves. The Cd immobilized by root cell walls was not transported into the cytoplasm, thereby protecting organelles and limiting Cd translocation to aerial parts of the plant (Fig. 3c, 3d). The present study indicates that 50 μ M B increased the proportion of cell wall-bound Cd and decreased the Cd distribution in plastids and organelles. In agreement with Riaz et al. (2021a), B reduced intracellular Cd transport by enhancing cell wall adsorption in rice. These results corresponded to the finding that the appropriate concentration of 50 μ M B

decreased Cd accumulation in aerial organs and alleviated Cd toxicity in *Malus hupehensis* Rehd. Seedlings (Fig. 2). Therefore, the enhanced Cd-binding capacity of root cell walls is recognized as an effective mechanism for improving Cd tolerance under 50 μM B exposure.

The Cd binding capacity of CWs depends on their composition (cellulose, pectin, and hemicellulose) and component properties. Under Cd stress, the 50 μM B significantly increased both the content and proportion of Cd in the pectin and HC1 fractions (Fig. 4a, b), indicating that pectin and HC1 play important roles in Cd immobilization. This result aligns with the findings of Wu et al. (2020c), in which B application enhanced Cd sequestration in the cell wall of *Brassica napus* by promoting its binding to pectin and cellulose. These results indicated that pectin-mediated Cd sequestration may constitute a key mechanism by which 50 μM B enhances the Cd-binding capacity of the cell wall. The CW metal binding capacity depends on the abundance of functional groups ($-\text{COO}$, $-\text{OH}$, and $\text{C}=\text{O}$). FTIR analysis revealed that 50 μM B increased the absorption intensities of these key groups (Fig. 4c). This increase in the number of reactive sites likely augmented the affinity of the CWs for Cd, further explaining the increased pectin – Cd binding (Yang et al., 2022). Additionally, 50 μM B further enhanced the Cd-induced increases in the contents of pectin contributing to improved Cd binding ability (Fig. 4d). More importantly, root PME activity was significantly increased under 50 μM B treatment in the presence of Cd (Figure S1). PME catalyzes the demethylation of pectin, generating negatively charged carboxyl groups that enhance the capacity for Cd^{2+} binding (Douchiche et al., 2010). Consistent with this mechanism, our results demonstrate that 50 μM B promotes the demethylation of highly methyl-esterified pectin under Cd exposure by enhancing root PME activity, thereby increasing the abundance of free carboxyl groups available for Cd binding.

Appropriate B enhanced tolerance to Cd stress in apple rootstock by activating the antioxidant defense system and regulating related gene expression

Cd stress induces reactive oxygen species (ROS) accumulation and membrane lipid peroxidation in plants, leading to oxidative stress (Gill and Tuteja, 2010). In this study, 50 μM B supply significantly decreased the levels of O_2^- , H_2O_2 , and MDA in both Cd-exposed roots and leaves (Fig. 5). These results demonstrated that exogenous B application effectively mitigates Cd-induced oxidative damage. Thus, optimal B supply plays a critical role in enhancing the oxidative stress tolerance of *Malus hupehensis* Rehd. under Cd stress.

Plants alleviate heavy metal toxicity by activating both enzymatic and non-enzymatic antioxidant systems (Singh et al., 2016). In the present study, the reduced levels of ROS and MDA under 50 μM B treatment were associated with a marked increase in the contents of free proline, AsA, and T-SH in the roots, stems, and leaves (Fig. S2), along with enhanced activities of SOD in roots and leaves, POD across all tissues, CAT in stems, and APX in roots (Fig. S3). The results indicated that *Malus hupehensis* Rehd. seedlings treated with 50 μM B exhibited greater antioxidant capacity compared with other B concentrations. These findings are consistent with earlier reports in pepper and rice (Riaz et al., 2021b,

Huang et al., 2021b), where exogenous B alleviated Cd-induced oxidative stress by strengthening the antioxidant defense system.

B application reduced Cd accumulation and enhanced Cd tolerance in *Malus hupehensis* Rehd., which is probably associated with B-modulated transcription of genes regulating Cd uptake and detoxification. *IRT1* has been shown to enhance Cd accumulation when overexpressed in *Arabidopsis thaliana* (Pedas et al., 2008). Under Cd stress, the expression of *ZIP6* and *IRT1* was markedly downregulated in plants treated with 50 μ M B, suggesting that B2 + Cd treatment effectively reduced Cd uptake. This finding aligns with the observed decrease in root Cd concentration under Cd stress following B2 + Cd treatment. Persans et al. (2001) reported that the vacuolar membrane protein MTP1 is involved in cytoplasmic Cd transport, with its expression level showing a significant positive correlation with vacuolar Cd accumulation capacity. In the present study, Cd stress significantly upregulated *MTP1* and *MHX* expression except for B-free treatment, but their expression levels were always lowest under B2 + Cd treatment (Fig. 6). These results indicate that under Cd stress, vacuolar compartmentalization significantly mitigates Cd toxicity in *Malus hupehensis* Rehd.. However, appropriate B supply does not enhance the vacuolar compartmentalization of Cd, suggesting that B likely reduces Cd toxicity primarily by strengthening the cell wall rather than via vacuolar sequestration. This hypothesis is consistent with the subcellular distribution results described above (Fig. 3c-d), which showed that under B2 treatment, the proportion of Cd in root vacuoles decreased while that in the cell wall increased. Together, these findings elucidate the molecular mechanism by which B modulates Cd detoxification.

5 Conclusion

As summarized in Fig. 7, application of B at an appropriate concentration (50 μ M) effectively alleviates Cd toxicity in *Malus hupehensis* Rehd. seedlings. The mitigation is achieved through multiple interconnected mechanisms: (1) B supply enhances Cd immobilization in the root cell wall by increasing pectin content and pectin methylesterase activity, thereby promoting the binding of Cd to cell wall components and altering its subcellular distribution toward less mobile and less toxic forms; (2) B activates both enzymatic and non-enzymatic antioxidant systems, reducing reactive oxygen species (ROS) accumulation and lipid peroxidation, thus improving cellular redox homeostasis under Cd stress. (3) B reduces Cd uptake by downregulating the expression of Cd influx transporters (*ZIP6* and *IRT1*). These findings reveal the pivotal role of B in enhancing Cd tolerance in apple rootstock through coordinated physiological responses, providing a theoretical basis for the application of B in mitigating Cd contamination in orchards and ensuring the sustainable production of fruit trees.

Declarations

Ethics approval and consent to participate

Not applicable. **Consent for publication**

Competing interests

The authors declare no competing interests.

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Author Contribution

Ying Tong: Writing - review & editing, Writing original draft, Software, Methodology, Formal analysis, Data curation, Conceptualization. Xiang Li: Writing - review & editing, Methodology. Mingze Xu: Writing - review & editing. Sijun Qin: Writing - review & editing, Funding acquisition. Deguo Lyu: Writing-review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. Jiali He: Writing - review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

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Not applicable.

Data Availability

All data produced or examined throughout this study are contained within this article. For additional inquiries, please contact the corresponding author.

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Figures

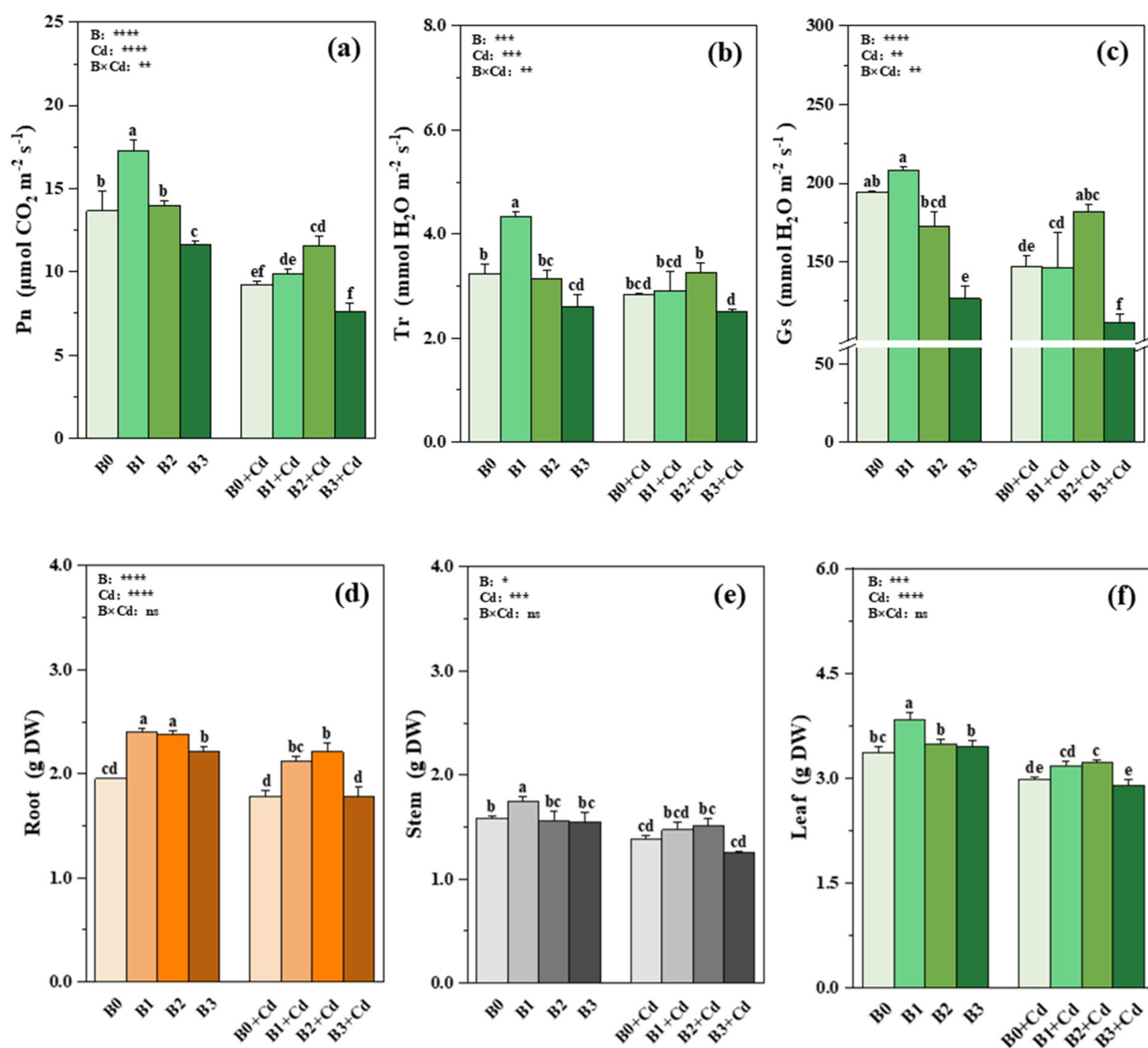


Figure 1

The photosynthetic parameters (a) (b) (c) and biomass of various plant tissues (d) (e) (f) of *Malus hupehensis* Rehd. exposed to 0 or 50 μM CdCl_2 combined with either 0, 12.5, 50 or 150 μM B for 30 days. Data are means $\pm$ standard error (SE; $n = 3$). Different letters after values indicate significant differences between treatments.

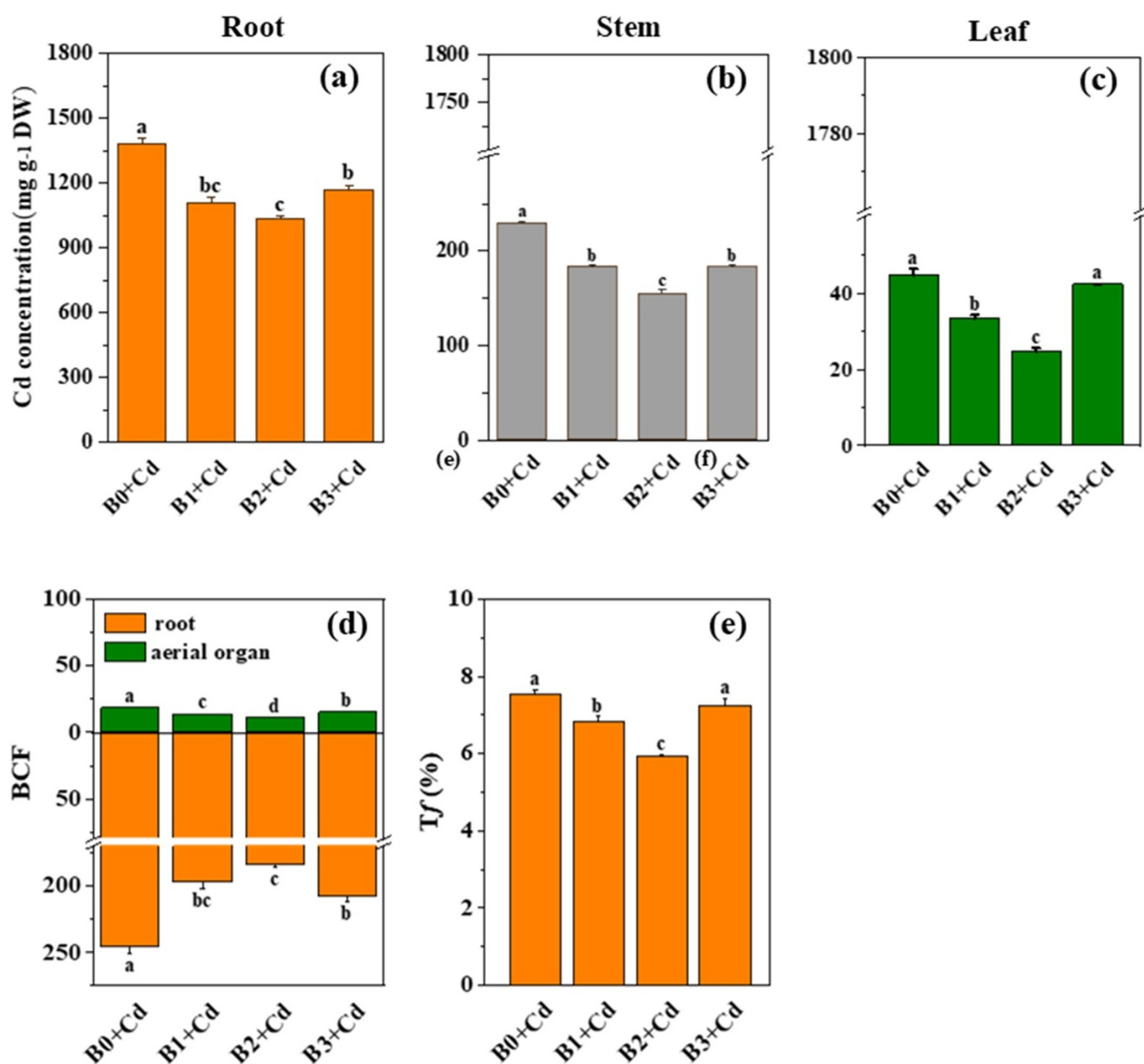


Figure 2

Cd content in *Malus hupehensis* Rehd. roots, stems, and leaves (a) (b) (c), accumulation coefficient, and translocation coefficient (c) (e) exposed to 0 or 50 μM CdCl_2 combined with either 0, 12.5, 50 or 150 μM B for 30 days. Data are means $\pm$ standard error (SE; $n = 3$). Different letters after values indicate significant differences between treatments.

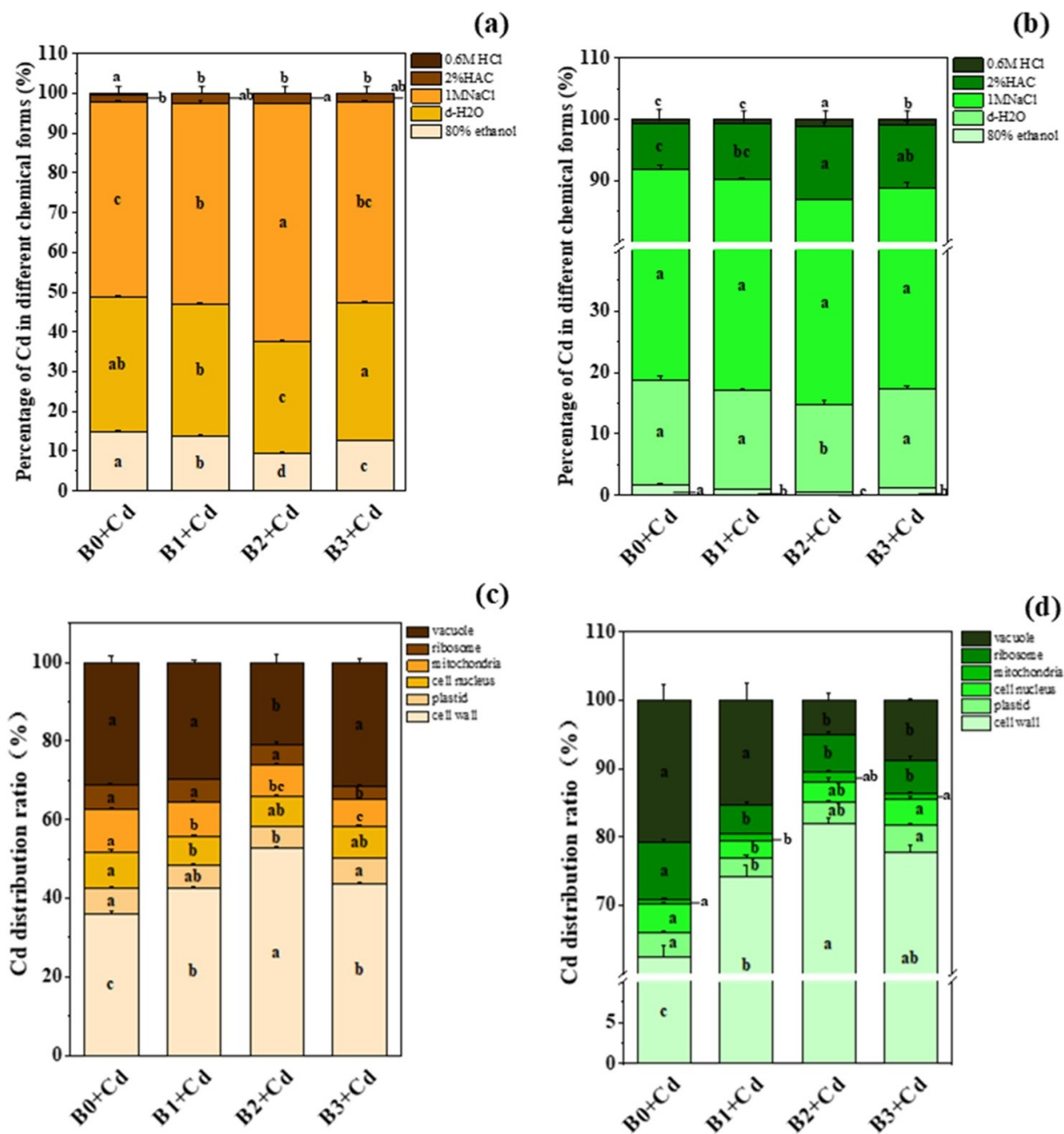


Figure 3

The subcellular Cd allocation ratio (a) (b) and different forms of Cd content and proportion (c) (d) in *Malus hupehensis* Rehd. roots and leaves exposed to 0 or 50 μM CdCl_2 combined with either 0, 12.5, 50 or 150 μM B for 30 days. Data are means $\pm$ standard error (SE; n = 3). Different letters after values indicate significant differences between treatments.

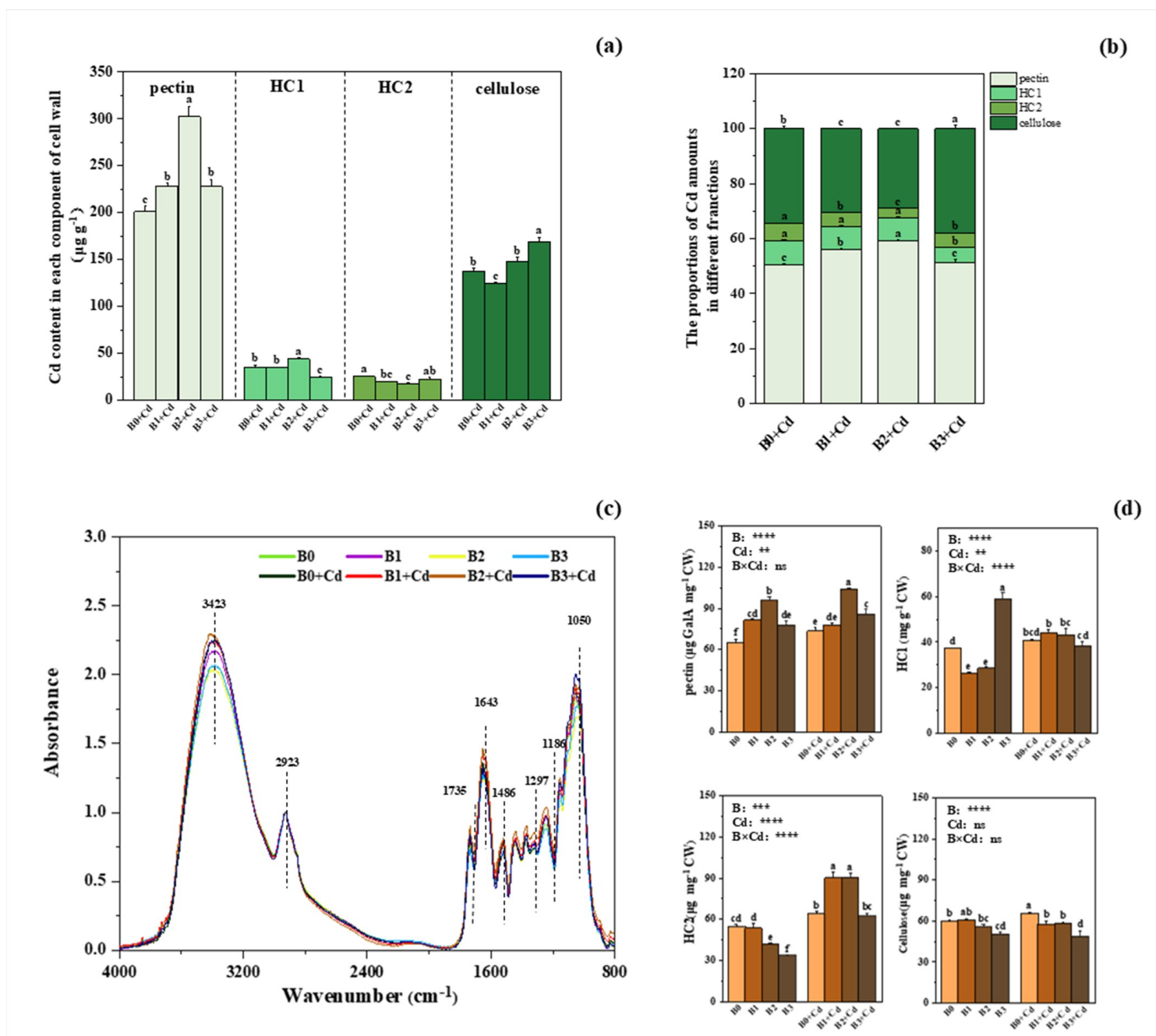


Figure 4

The Cd content (4a) and the proportion of Cd (4b) in each component of root cell walls, infrared spectral characteristics of root cell walls (4c) and the content of each component of root cell wall (4d) in *Malus hupehensis* Rehd. exposed to 0 or 50 μM CdCl_2 combined with either 0, 12.5, 50 or 150 μM B for 30 days. Data are means $\pm$ standard error (SE; $n = 3$). Different letters after values indicate significant differences between treatments.

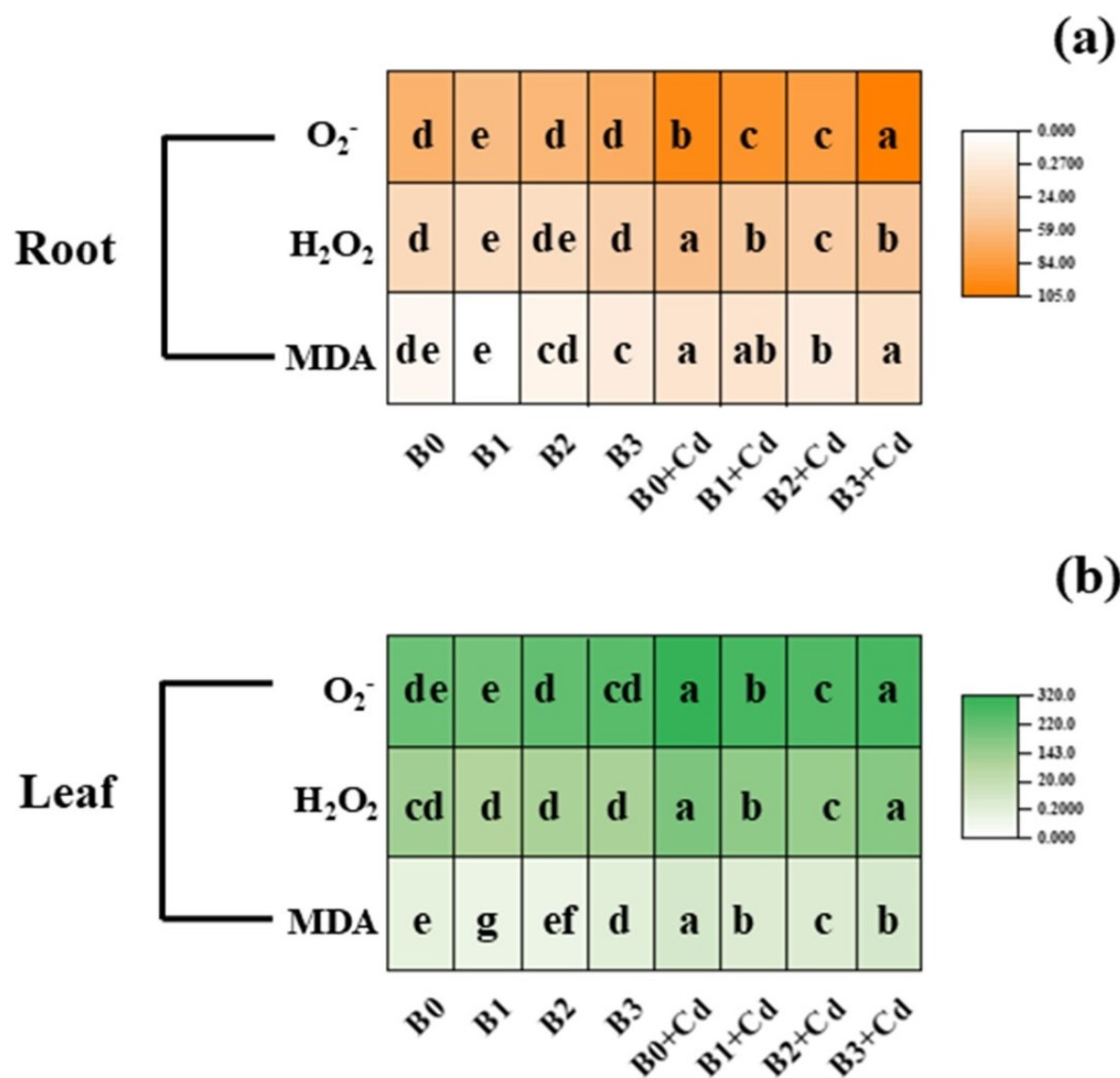


Figure 5

The contents of O_2^- , H_2O_2 , and MDA in roots and leaves of *Malus hupehensis* Rehd. exposed to 0 or 50 μM CdCl_2 combined with either 0, 12.5, 50 or 150 μM B for 30 days. Data are means $\pm$ standard error (SE; $n = 3$). Different letters after values indicate significant differences between treatments.

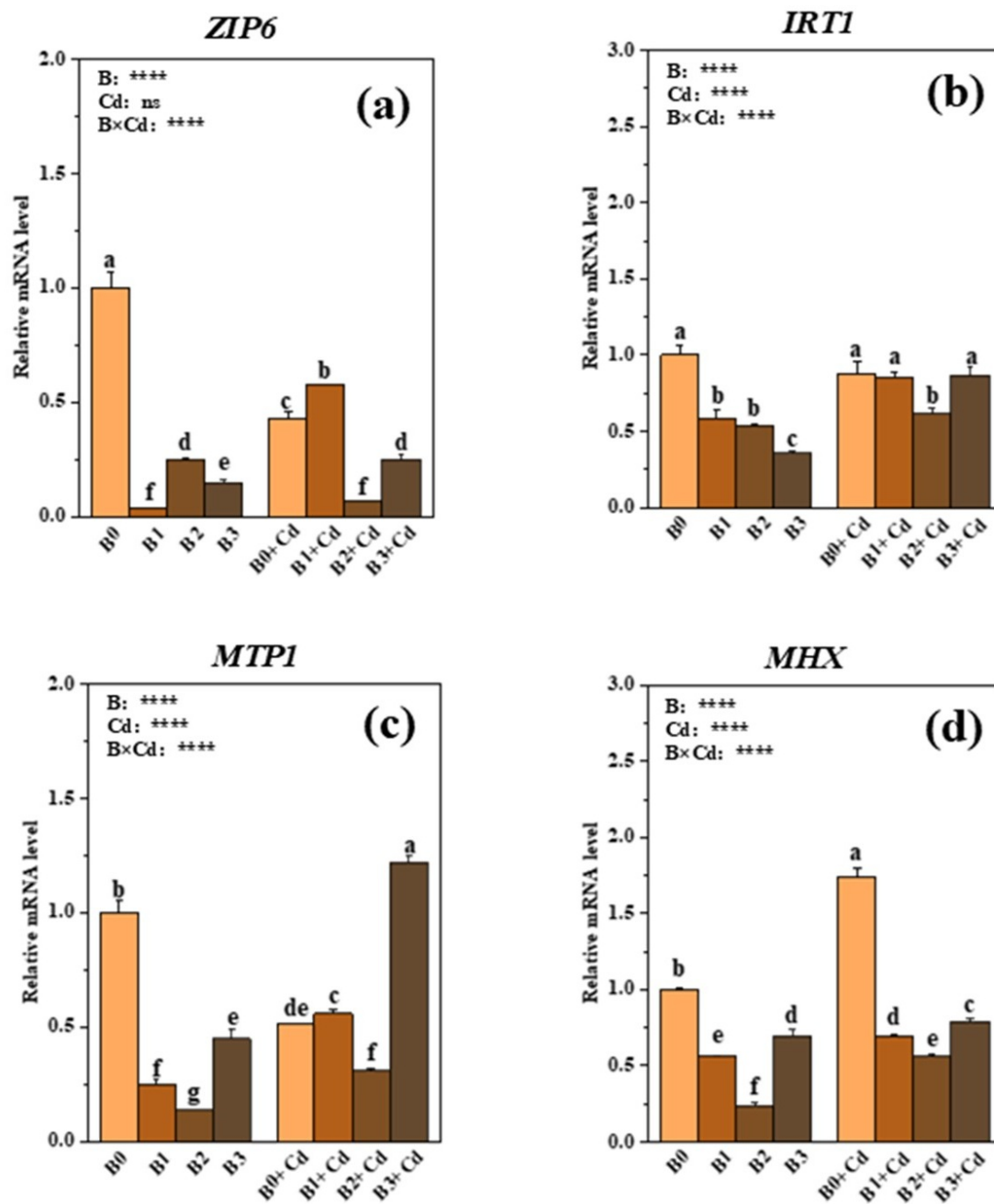


Figure 6

Expression of genes involved in Cd absorption and transport exposed to 0 or 50 μM CdCl_2 combined with either 0, 12.5, 50 or 150 μM B for 30 days. Data are means $\pm$ standard error (SE; $n = 3$). Different letters after values indicate significant differences between treatments.

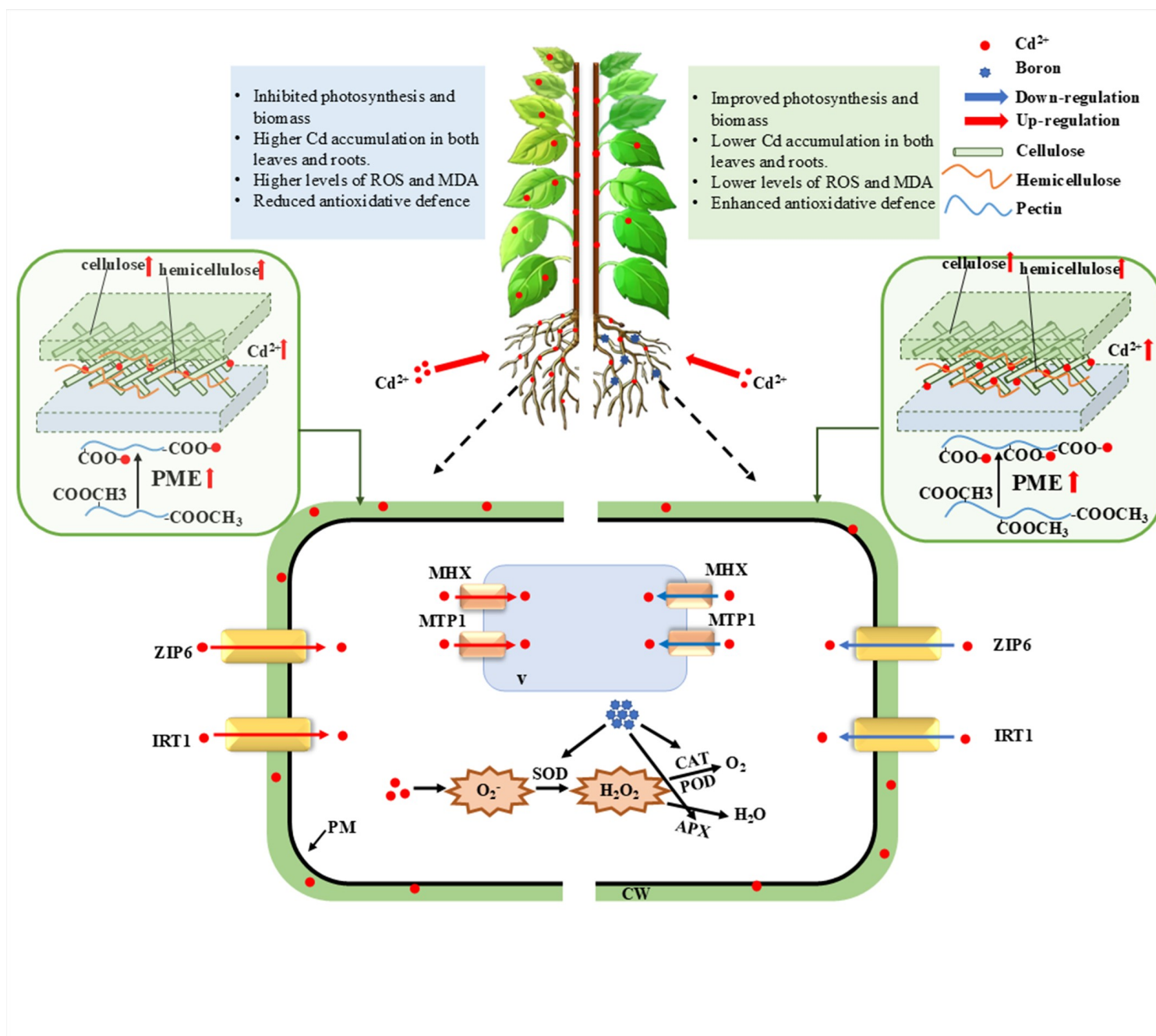


Figure 7

Schematic model of B-mediated mitigation of Cd toxicity in *Malus hupehensis* Rehd. exposed to 50 μM CdCl_2 (Cd) or 50 μM CdCl_2 (Cd) together with 50 μM H_3BO_3 for a designated period. Cd^{2+} uptake and accumulation in Cd-exposed seedlings (left) and B-treated seedlings under Cd exposure (right).

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