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Foliar Application of Selenium Reduces Cadmium Accumulation in Walnut Seedlings

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Abstract: Cadmium (Cd) and selenium (Se) could jointly affect plant growth. To investigate the affect of Se on the Cd accumulation in *Juglans regia* and the physiological mechanism by which Se regulates Cd-induced oxidative stress, in this study, the effects of different foliar application doses of Se (0 (Se0), 20 (Se20), and 200 (Se200) μM) on *J. regia* (variety Xinfeng) seedlings under Cd stress (5 mM) were determined. The results show that exogenous application of Se (Se20 and Se200) increased walnut biomass compared with Se0 under Cd stress. Under Cd stress, exogenous application of 20 μM Se increased the catalase (CAT), peroxidase (POD), and ascorbate oxidase (AAO) activities in walnut roots and the CAT and AAO activities in walnut leaves, and exogenous application of 200 μM Se increased the CAT, POD, and AAO activities in walnut roots. Furthermore, under Cd stress, exogenous application of 20 and 200 μM Se both decreased the contents of superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) in walnut roots and the content of MDA in walnut leaves. Moreover, application of 20 and 200 μM Se both reduced the accumulation of Cd in the root, wood, bark, and leaves of walnuts, and application of 200 μM Se enhanced Se concentration in the root, wood, bark, and leaves. Overall, exogenous application of Se, especially 200 μM Se, could reduce Cd accumulation and enhance CAT, POD, and AAO activities in Cd-stressed walnut roots, thus alleviating Cd stress. This study provides technical guidance for reducing the effects of Cd stress on walnut growth.

Keywords: *Juglans regia*; biomass; soil contamination; oxidative stress; antioxidant enzymes

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1. Introduction

About 30,000 tons of cadmium (Cd) are released into the environment every year worldwide due to human activities, such as mining, the use of pesticides and fertilizers, and the combustion of fossil fuels [1]. Cd in soil can be absorbed by plant roots and transported to the aerial parts through the xylem, which inevitably causes ion imbalance, lowered photosynthetic capacity, and oxidative stress in plants [2]. Therefore, Cd stress always reduces agricultural productivity, and threatens human health via food chains [1,3–6]. Previous studies estimated that more than 80% of the Cd accumulated in human bodies is from vegetables and crops grown in Cd-contaminated soils [7]. A recent survey report on soil contamination in China shows that the area of Cd-contaminated soil ranks first among the heavy metal-contaminated soils, accounting for 7% [8]. At present, Cd is detected in rice [9], peanuts [10], apples [6], pears, grape, peach-shaped plums, and oranges [3].

Walnut (*Juglans regia* L.) is one of the four major nuts in the world. Due to the high nutrition content and economic value, walnut is widely cultivated in Asia, Europe, America, South Africa, Australia, and New Zealand [11,12]. However, Peng found that soil Cd contamination greatly affected walnut growth and yield [13]. Previous studies show that the highest Cd concentration in walnut kernels reaches 0.71 mg kg^{-1}, which exceeds the safety standard [14–17].

Selenium (Se) is widely distributed worldwide. However, there are significant differences in the concentration and chemical forms of Se in natural soils in different regions [18,19]. Se is a non-essential element for plants, but an appropriate amount of Se could promote plant growth. Several studies found that Se can increase chloroplast size, enhance chloroplast ultrastructure [20] and antioxidant capacity [21], as well as regulate the uptake and translocation of essential elements [22], improving crop yield and quality [23]. Moreover, some studies also show that Se could reduce Cd accumulation and reactive oxygen species (ROS) contents by regulating carbon and nitrogen metabolism in potatoes [7,24,25]. However, Yu et al. found that foliar application of Se enhanced the accumulation of Cd in tobacco [26]. Yu et al. reported that Se application decreased Cd concentration in *Brassica chinensis* shoots treated with 10 μM Cd, but increased the Cd concentration in shoots treated with 50 μM Cd [27]. Therefore, the application of different concentrations of Se have different effects on Cd accumulation in plants [27]. This may be caused by the differences in plant species, Se concentration, Se application method, and Cd dosage. However, the effect of different application rates of Se on Cd accumulation in walnut is unknown at present.

Besides, Se is an essential mineral element for both humans and animals [28]. Studies show that Se deficiency is associated with Keshan disease (KD) and white muscle disease [29]. According to the report of the World Health Organization (WHO), China is one of the 40 Se-deficient countries in the world [30]. More than half of China's land is deficient in Se, and over 105 million people's health is threatened by Se deficiency [19]. Therefore, improving the Se concentration in foods is urgent for improving the health of Chinese people.

Previous studies show that exogenous application of appropriate amount of Se may reduce Cd accumulation and increase Se concentration in rice under Cd stress [31]. Therefore, in this study, the effects of different doses of Se (0, 20, and 200 μM) on ROS contents, antioxidant enzyme activities, and Cd accumulation in different organs of *J. regia* (variety Xinfeng) were analyzed under Cd stress (5 mM), aiming to clarify the physiological mechanism by which Se regulates Cd-induced oxidative stress. We hypothesized that: (1) Se application might reduce Cd accumulation in walnut; and (2) Se application might cause physiological responses of walnut that reduce oxidative stress caused by Cd. The study will provide guidance for reducing Cd concentration and increasing Se concentration in walnuts through agronomic measures to provide high-quality Se-enriched walnuts.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The experiments were conducted at Henan Agricultural University, Zhengzhou, China (34°47′8″ N, 113°39′50″ E). Seedlings of *Juglans regia* (variety Xinfeng) were cultivated in a greenhouse (day/night temperature: 35/25 °C; relative humidity: 50–60%; natural light). After germination, seedlings were planted in plastic pots filled with sands, and the quarter-strength Hoagland solution (100 mL) was added to each pot every three days. After 12 weeks, 36 seedlings with a height of about 25 cm were selected, and divided equally into six groups.

Subsequently, walnut plants in the six groups were exposed to 0 (-Cd) or 5 (+Cd) mM $CdCl_2$ together with 0 (Se0), 20 (Se20), or 200 (Se200) μM Na_2SeO_3. Briefly, this experiment contained six treatments including Se0Cd0 (0 μM Na_2SeO_3 + 0 mM $CdCl_2$), Se0Cd5 (0 μM Na_2SeO_3 + 5 mM $CdCl_2$), Se20Cd0 (20 μM Na_2SeO_3 + 0 mM $CdCl_2$), Se20Cd5 (20 μM Na_2SeO_3 + 5 mM $CdCl_2$), Se200Cd0 (200 μM Na_2SeO_3 + 0 mM $CdCl_2$), and Se200Cd5 (200 μM Na_2SeO_3 + 5 mM $CdCl_2$) (Table 1). Cd and Se doses were determined according to our pre-experiment and previous studies [27,31,32]. Our pre-experiment results show that walnuts cultured in sand could tolerate higher concentrations of Cd than walnuts cultured in nutrient solution. Furthermore, in previous studies, the Se concentrations for treating herbaceous plants were 3–20 μM [27,31]. For woody plants, such as walnut, the Se concentration should be higher. One hundred milliliters of quarter-strength Hoagland solution were added every three days. For Se0Cd5, Se20Cd5, and Se200Cd5 treatments,

Cd was supplied every three days with the nutrient solution. Exogenous Se was sprayed on walnut leaves after being dissolved in distilled water every three days. Seedlings in Se0Cd0 and Se0Cd5 treatments were sprayed with distilled water.

Table 1. Experimental design.

Treatment	Se (μM)	Cd (mM)
Se0Cd0	0	0
Se0Cd5	0	5
Se20Cd0	20	0
Se20Cd5	20	5
Se200Cd0	200	0
Se200Cd5	200	5

After a 60-day cultivation, the root system of each plant was washed carefully using 50 mM $CaCl_2$ for 3 min to remove Cd^{2+}. The shoots were rinsed with distilled water three times. Then, each plant was separated into root, wood, bark, and leaves. The fresh weight of each sample was recorded before wrapping with tinfoil and freezing in liquid nitrogen. The frozen samples were ground into fine powder in liquid nitrogen and stored at −80 °C for further analysis. About 150 mg powder of each organ of each plant was dried at 70 °C to determine the fresh-to-dry mass ratio, which was used to calculate the biomass of each organ.

2.2. Determination of Cd and Se

One hundred and fifty milligrams of powder of each organ were digested in a mixture containing 8 mL of concentrated HNO_3 and 2 mL of concentrated $HClO_4$ at 170 °C [33]. The Cd and Se concentrations in the extract were determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent7800, Santa Clara, CA, USA).

2.3. Analysis of $O_2^{\bullet -}$, H_2O_2, and MDA

The superoxide ($O_2^{\bullet -}$) content in the roots and leaves of the Xinfeng walnut were determined spectrophotometrically at 530 nm as described by Lei [34]. Briefly, 100 mg of powder was extracted in potassium phosphate buffer (50 mM, pH 7.8) and centrifuged (10,000× *g*, 4 °C, 10 min). Then, the supernatant was mixed with the potassium phosphate buffer (0.9 mL, 50 mM, pH 7.8) and hydroxylamine hydrochloride (0.1 mL, 10 mM). The mixture was then incubated at 25 °C for 20 min, before adding p-aminobenzene sulfonic acid (1 mL, 17 mM) and α-naphthylamine (1 mL, 7 mM). The absorbance of the mixture was determined at 530 nm after an incubation at 25 °C for 20 min.

The hydrogen peroxide (H_2O_2) contents in the roots and leaves were determined according to the method of He [35]. Briefly, 50 mg of powder was extracted in 5% trichloroacetic acid (TCA) for 20 min and centrifuged (10,000× *g*, 4 °C, 10 min). Then, the supernatant was mixed with 20% $TiCl_4$ and 25% aqueous ammonia. After that, the precipitate was collected and dissolved in H_2SO_4 (1 M). The absorbance of the solution was determined spectrophotometrically at 410 nm.

The malondialdehyde (MDA) contents in the roots and leaves were determined according to the method of Lei [34]. Briefly, 100 mg of powder was extracted in 10% TCA for 30 min and centrifuged (10,000× *g*, 4 °C, 10 min). Then, the supernatant was mixed with 0.6% thiobarbituric acid (TBA) and 10% TCA. After reacting in boiling water for 15 min, the mixture was rapidly cooled in ice water and centrifuged (10,000× *g*, 4 °C, 10 min). The absorbance of the solution was determined spectrophotometrically at 450, 532, and 600 nm.

2.4. Assays of Antioxidant Enzyme Activities

The soluble proteins in the roots and leaves were extracted based on the method of Luo [36]. Briefly, frozen powder was homogenized in a cold extraction buffer containing 100 mM potassium phosphate (pH 7.8), 200 mg polyvinylpolypyrrolidone, and 0.5% (*v*/*v*) Triton X-100. Then, the mixture was incubated for 15 min in an ice bath and centrifuged

(15,000× g, 4°C, 30 min). The supernatant was eluted through Sephadex G-25 columns (PD-10 column, Pharmacia, Freiburg, Germany). The soluble proteins in the eluent were determined according to the Bradford method, using bovine serum albumin (Interchim, Montluçon, France) as the standard. The soluble protein extracts were used for the assays of enzyme activities.

The activity of catalase (CAT, EC 1.11.1.6) was measured according to the method of He [37], and the absorbance of the reaction system was determined spectrophotometrically at 240 nm. The reaction system contained 50 mM potassium phosphate buffer (pH 7.0), 40 mM H_2O_2, and the protein extract. One unit of CAT was defined as the amount of the enzyme that is needed to decompose 1 mmol of H_2O_2 per min at 25 °C.

The activity of peroxidase (POD, EC 1.11.1.7) was determined according to the method of Chen [38], and the absorbance of the reaction system was determined spectrophotometrically at 436 nm. The reaction system contained 50 mM potassium phosphate buffer (pH 6.5), 40 mM guaiacol, 10 mM H_2O_2, and the protein extract. One unit of the enzyme was defined as the amount of POD that is needed to oxidize 1 mmol of guaiacol min^{-1} mg^{-1} protein.

The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured according to the method of He [37], and the absorbance of the reaction system was determined spectrophotometrically at 290 nm. The reaction system contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1 mM sodium ascorbate, 2.5 mM H_2O_2, and the protein extract. One unit of APX was defined as the amount of the enzyme that is needed to degrade 1 μmol of ascorbate min^{-1} mg^{-1} protein.

The activity of ascorbate oxidase (AAO, EC 1.10.3.3) was determined according to the method of Tamás [39], and the absorbance of the reaction system was determined spectrophotometrically at 265 nm. The reaction system contained 100 mM potassium phosphate buffer (pH 5.6), 5 mM EDTA-Na_2, 50 mM sodium ascorbate, and the protein extract. One unit of AAO was defined as the amount of the enzyme that is needed to oxidize 1 μmol of ascorbate min^{-1} mg^{-1} protein.

2.5. Statistical Analysis

Data were analyzed using Statgraphics (STN, St Louis, MO, USA). Two-way analysis of variances (ANOVA) was performed to determine the significant differences. If it was significant, a posteriori comparison of means was performed. The normality of the data was tested before statistical analysis. Differences between means were considered significant when the p-value was less than 0.05 according to the ANOVA F-test.

3. Results

3.1. Effect of Se and Cd on the Growth of Xinfeng Walnut

Under Cd-free condition, the application of 20 μM Se increased the biomass of wood, bark, and leaves (Table 2). The biomass of the wood, bark, and leaves in the Se20Cd0 treatment was 4.02, 2.76, and 12.65 g, respectively, which was 20%, 17%, and 18% higher than those in the Se0Cd0 treatment, respectively, and there was no difference in root biomass (Table 2). However, under Cd-free condition, the application of 200 μM Se inhibited the root and leaf growth of Xinfeng walnut (Table 2). The biomass of roots and leaves in the Se200Cd0 treatment was 9.58 and 8.96 g, respectively, which was 13% and 17% lower than those in the Se0Cd0 treatment, respectively, and there was no difference in the biomass of wood and bark (Table 2).

Table 2. The biomass of the roots, wood, bark, and leaves of *J. regia* (variety Xinfeng) in different treatments.

Se (μM)	Cd (mM)	Root (g)	Wood (g)	Bark (g)	Leaves (g)
0	0	10.99 ± 0.24 d	3.34 ± 0.12 b	2.35 ± 0.13 b	10.79 ± 0.62 c
	5	6.37 ± 0.30 a	2.99 ± 0.24 ab	1.96 ± 0.07 a	7.16 ± 0.12 a
20	0	11.25 ± 0.80 d	4.02 ± 0.15 c	2.76 ± 0.14 c	12.65 ± 0.42 d
	5	8.26 ± 0.30 b	2.79 ± 0.24 a	2.01 ± 0.07 a	7.86 ± 0.94 ab
200	0	9.58 ± 0.39 c	3.18 ± 0.13 ab	2.17 ± 0.09 ab	8.96 ± 0.27 b
	5	9.63 ± 0.42 c	3.11 ± 0.13 ab	2.14 ± 0.10 ab	8.86 ± 0.36 b
p-values	Cd	****	***	****	****
	Se	*	ns	*	*
	Cd × Se	****	**	**	***

Notes: Data were means ± SE (n = 6). Different lowercase letters in the same column indicate significant difference between treatments. *p*-values of the ANOVAs for Cd, Se, and their interaction (Cd × Se) are also indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; and ns, insignificant.

The biomass of the roots, wood, bark, and leaves was lower in the Se0Cd5 and Se20Cd5 treatments than in the Se0Cd0 and Se20Cd0 treatments, respectively (Table 2), and there was no difference between Se200Cd5 treatment and Se200Cd0 treatment (Table 2). Under Cd stress, the application of Se (Se20 and Se200) increased the biomass of the roots and leaves of the Xinfeng walnut (Table 2). The biomass of the root and leaves in the Se20Cd5 treatment was 8.26 and 7.86 g, respectively, which were 30% and 10% higher than those in the Se0Cd5 treatment, respectively (Table 2). The biomasses of the roots and leaves in the Se200Cd5 treatment were 9.63 and 8.86 g, respectively, which were 51% and 24% higher than those in the Se0Cd5 treatment, respectively (Table 2).

3.2. The Se and Cd Accumulations in Walnuts

Cd was not detected in the walnuts without Cd exposure (Figure 1a–d). The Cd concentrations in root and wood in the Se20Cd5 treatment were 1782.80 and 12.58 μg g^{-1} dry weight, respectively, which were 12% and 14% lower than those in the Se200Cd5 treatment, respectively (Figure 1a,b). Furthermore, there was no difference in the Cd concentration in the bark and leaves between Se20Cd5 treatment and Se0Cd5 treatment (Figure 1c,d). The Cd concentrations in the root, wood, bark, and leaves in the Se200Cd5 treatment were 1526.33, 10.28, 347.46, and 34.42 μg g^{-1} dry weight, respectively, which were 25%, 26%, 21%, and 23% lower than those in the Se0Cd5 treatment, respectively (Figure 1a,d).

Under Cd exposure or Cd-free conditions, the application of 20 μM Se increased Se concentration in bark and leaves, but did not alter Se concentration in root and wood compared with Se0 (Figure 1e–h). Under Cd-exposure or Cd-free conditions, the application of 200 μM Se increased the Se concentration in root, wood, bark, and leaves, compared with Se0 (Figure 1e–h).

3.3. Effects of Se Application on $O_2^{\bullet -}$, H_2O_2, and MDA Contents in Xinfeng Walnut

The $O_2^{\bullet -}$ contents in the roots of the Xinfeng walnut in the Se0Cd5, Se20Cd5, and Se200Cd5 treatments were 6.48, 5.72, and 5.28 μmol g^{-1} dry weight, respectively, which were 13%, 10%, and 21% higher than that in the Se0Cd0, Se20Cd0, and Se200Cd0 treatments, respectively, and the root $O_2^{\bullet -}$ content in the Se20Cd5 and Se200Cd5 treatments was 5.72 and 5.28 μmol g^{-1} dry weight, respectively, was were 12% and 8% lower than that in the Se0Cd5 treatment (Figure 2a). The leaf $O_2^{\bullet -}$ content in the Se0Cd5 treatment was 27% higher than that in the Se0Cd0 treatment, and the leaf $O_2^{\bullet -}$ content in the Se20Cd5 and Se200Cd5 treatments was 16% and 26% lower than that in the Se0Cd5 treatment (Figure 2b).

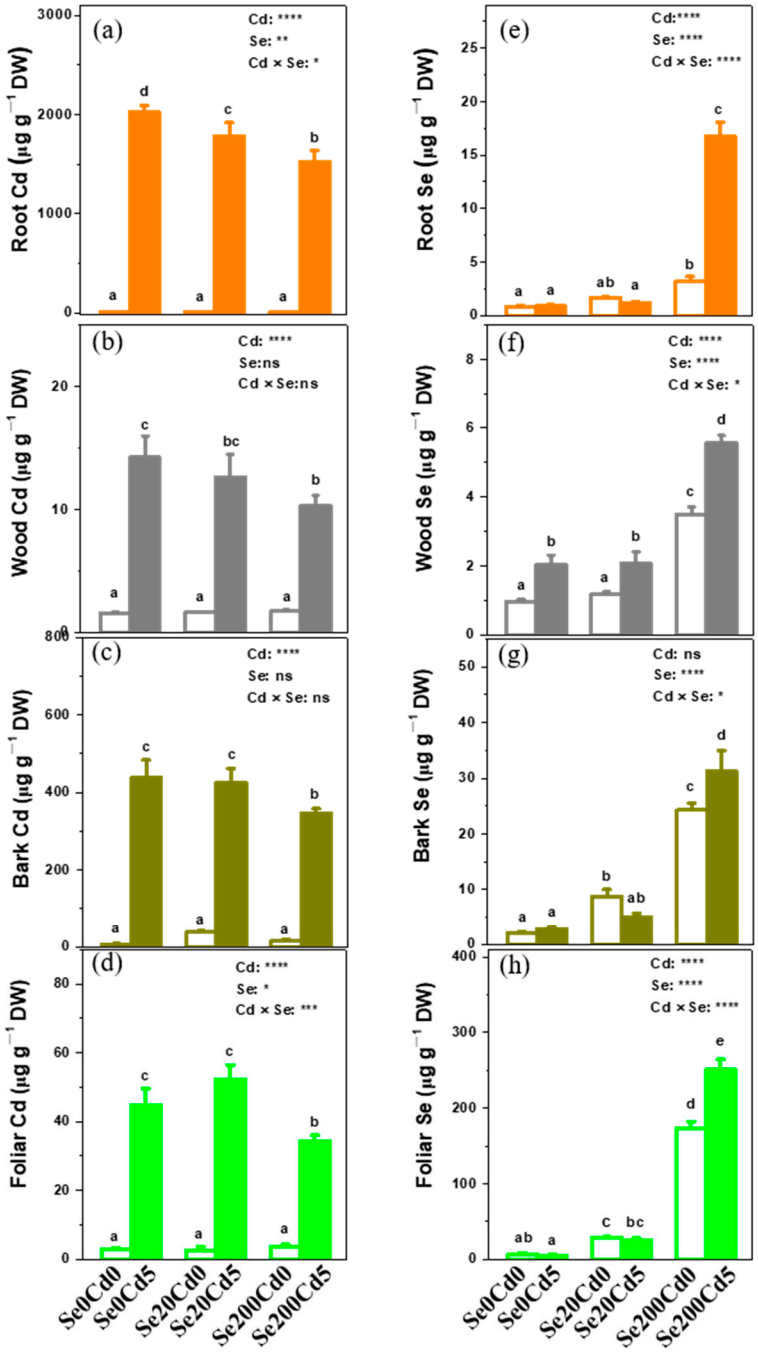

Figure 1. The Cd (**a**–**d**) and Se (**e**–**h**) concentrations in the roots, wood, bark, and leaves of *J. regia* (variety Xinfeng) exposed to either 0 (Cd0) or 5 mM (Cd5) Cd together with one of three Se levels (0 (Se0), 20 (Se20), or 200 (Se200) μM Se). Data were means ± SE (n = 6). Different lowercase letters on the bars indicate significant difference at $p < 0.05$ (F-test). *p*-values of the ANOVAs for Cd, Se, and their interaction (Cd × Se) are also indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; and ns, insignificant.

The root H_2O_2 content in the Se0Cd5, Se20Cd5, and Se200Cd5 treatments was 473.22, 365.60, and 345.35 μmol g^{-1} dry weight, respectively, which was 38%, 35%, and 27% higher than that in the Se0Cd0, Se20Cd0, and Se200Cd0 treatments, respectively (Figure 2c). The root H_2O_2 content in the Se20Cd5 and Se200Cd5 groups was 365.60 and 345.35 μmol g^{-1} dry weight, respectively, which was 23% and 27% lower than that in the Se0Cd5 treatment, respectively (Figure 2c). The leaf H_2O_2 content in the Se0Cd5 treatment was 164.63 μmol g^{-1} dry weight, which was 35% higher than that in the Se0Cd0 treatment

(Figure 2d). The leaf H_2O_2 content in the Se20Cd5 and Se200Cd5 treatments had no difference with that in the Se0Cd5 treatment (Figure 2d).

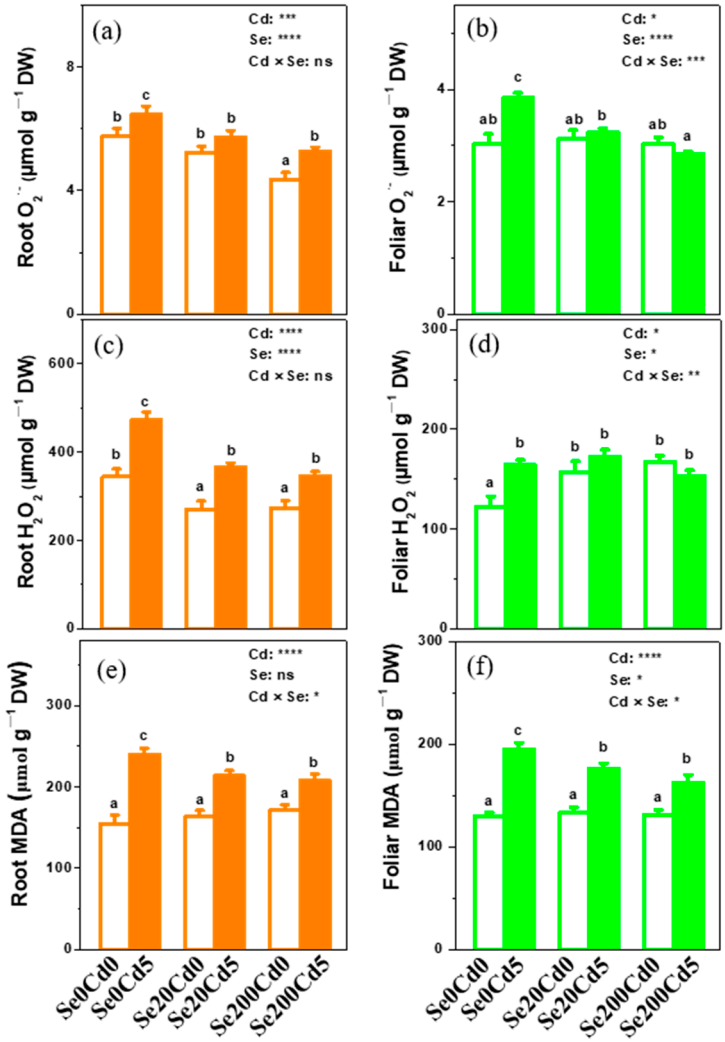

Figure 2. The contents of $O_2^{\bullet-}$ (**a**,**b**), H_2O_2 (**c**,**d**), and MDA (**e**,**f**) in the roots and leaves of *J. regia* (variety Xinfeng) exposed to either 0 (Cd0) or 5 mM (Cd5) Cd together with one of three Se levels (0 (Se0), 20 (Se20) or 200 (Se200) μM Se). Data were means ± SE (n = 6). Different lowercase letters on the bars indicate significant difference at $p < 0.05$ (F-test). *p*-values of the ANOVAs for Cd, Se and their interaction (Cd × Se) are also indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; and ns, insignificant.

The root MDA content in the Se0Cd5, Se20Cd5, and Se200Cd5 treatments was 240.21, 213.60, and 208.01 μmol g^{-1} dry weight, respectively, which was 55%, 31%, and 21% higher than those in the Se0Cd0, Se20Cd0, and Se200Cd0 treatments, respectively (Figure 2e). The root MDA content in the Se20Cd5 and Se200Cd5 treatments was 213.60 and 208.01 μmol g^{-1} dry weight, respectively, which was 11% and 13% lower than that in the Se0Cd5 treatment, respectively (Figure 2e). The leaf MDA content in the Se0Cd5, Se20Cd5, and Se200Cd5 treatments was 194.62, 175.99, and 162.23 μmol g^{-1} dry weight, respectively, which was 50%, 32%, and 24% higher than those in the Se0Cd0, Se20Cd0, and Se200Cd0 treatments, respectively (Figure 2f). The leaf MDA content in the Se20Cd5 and Se200Cd5 treatments was 175.99 and 162.23 μmol g^{-1} dry weight, respectively, which was 10% and 17% lower than that in the Se0Cd5 treatment, respectively (Figure 2f). However, there were no differences in root and leaf MDA contents between Se20Cd5 treatment and Se200Cd5 treatment (Figure 2e,f).

3.4. Effects of Se Application on the Antioxidant Enzyme Activities in Xinfeng Walnut

The root CAT activity in the Se0Cd5 treatment was lower than that in the Se0Cd0 treatment (Figure 3a). The root CAT activity in the Se20Cd5 and Se200Cd5 treatments was 302.26 and 220.86 U g^{-1} protein, respectively, which was 127% and 66% higher than that in the Se0Cd5 treatment (Figure 3a). The leaf CAT activity in the Se0Cd5 treatment was 46.47 U g^{-1} protein, which was 33% lower than that in the Se0Cd0 treatment (Figure 3b). The leaf CAT activity in the Se20Cd5 and Se200Cd5 treatments was 78.96 and 72.00 U g^{-1} protein, respectively, which was 77% and 55% higher than that in the Se0Cd5 treatment, respectively (Figure 3b).

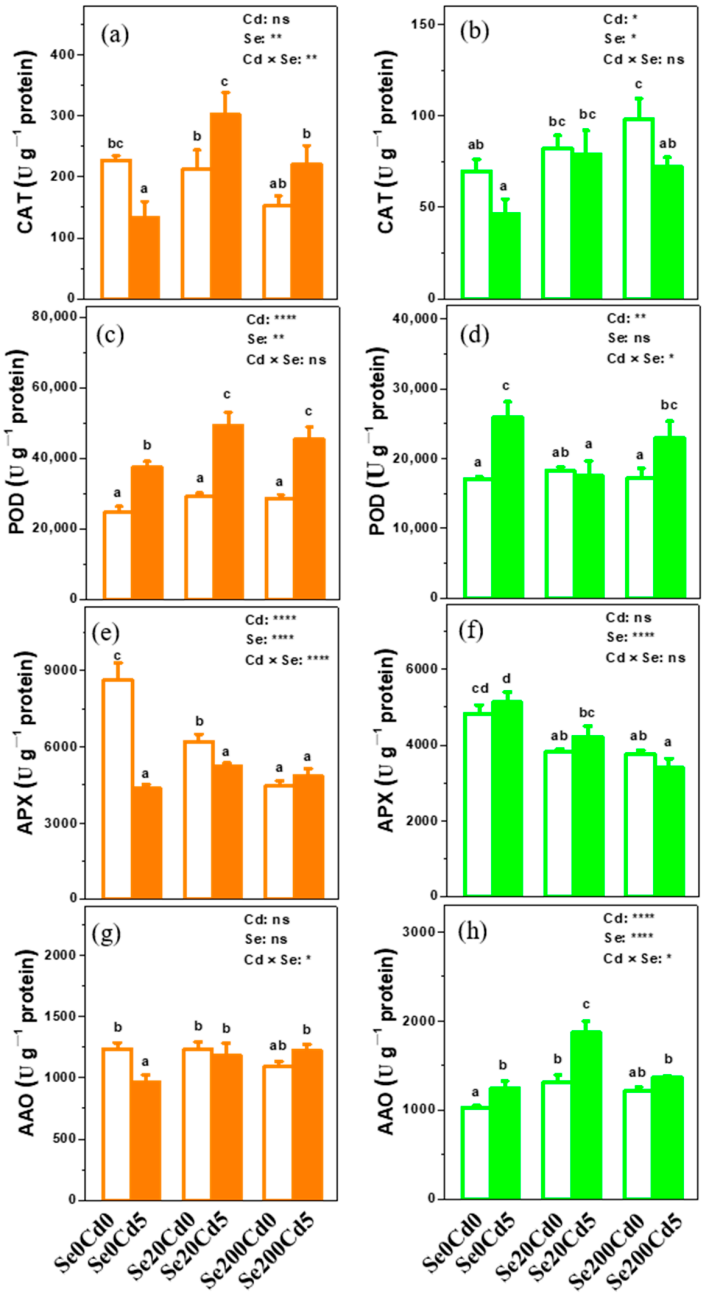

Figure 3. The activities of CAT (**a**,**b**), POD (**c**,**d**), APX (**e**,**f**), and AAO (**g**,**h**) in the roots and leaves of Xinfeng walnut exposed to either 0 (Cd0) or 5 mM (Cd5) Cd together with one of three Se levels (0 (Se0), 20 (Se20), or 200 (Se200) μM Se). Data were means ± SE (n = 6). Different lowercase letters on the bars indicate significant difference at $p < 0.05$ (F-test). *p*-values of the ANOVAs for Cd, Se and their interaction (Cd × Se) are also indicated. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; and ns, insignificant.

The root POD activity in the Se0Cd5 treatment was 37,324.80 U g^{-1} protein, which was 51% higher than that in the Se0Cd0 treatment, and the root POD activity in the Se20Cd5 and Se200Cd5 treatments was 49,309.73 and 45,284.17 U g^{-1} protein, respectively, which was 21% and 32% higher than that in the Se0Cd5 treatment, respectively (Figure 3c). The leaf POD activity in the Se0Cd5 treatment was 25,872.33 U g^{-1} protein, which was 52% higher than that in the Se0Cd0 treatment (Figure 3c), and the leaf POD activity in the Se20Cd5 treatment was 17,489.86 U g^{-1} protein, which was 32% lower than that in the Se0Cd5 treatment (Figure 3d). There was no difference in the leaf POD activity between Se200Cd0 and Se0Cd0 treatments and between Se20Cd0 and Se0Cd0 treatments (Figure 3d).

The root APX activity in the Se0Cd5 treatment was 4359.23 U g^{-1} protein, which was 50% lower than that in the Se0Cd0 treatment (Figure 3e), and there was no difference in the root APX activity between Se20Cd5 and Se200Cd5 treatments (Figure 3e). There was also no difference in the leaf APX activity between Se0Cd5 treatment and Se0Cd0 treatment (Figure 3f). The leaf APX activities in the Se20Cd5 and Se200Cd5 treatments were 4225.99 and 3406.56 U g^{-1} protein, respectively, which were 18% and 34% lower than that in the Se0Cd5 treatment, respectively (Figure 3f).

The root AAO activity in the Se0Cd5 treatment was 968.49 U g^{-1} protein, which was 22% lower than that in the Se0Cd0 treatment. The leaf AAO activity in the Se0Cd5 treatment was 1243.63 U g^{-1} protein, which was 22% higher than that in the Se0Cd0 treatment (Figure 3g,h). The root AAO activities in the Se20Cd5 and Se200Cd5 treatments were 1182.46 and 1224.03 U g^{-1} protein, which were 22% and 26% higher than that in the Se0Cd5 treatment, respectively (Figure 3g). The leaf AAO activity in the Se20Cd5 treatment was 1870.53 U g^{-1} protein, which was 50% higher than that in the Se0Cd5 treatment, but there was no difference between Se200Cd5 treatment and Se0Cd5 treatment (Figure 3h).

4. Discussion

4.1. Se Application Promoted Xinfeng Walnut Growth and Reduced Cd Accumulation

Se is beneficial to plant growth. An appropriate dosage of Se could promote plant growth and increase crop yield [7,23]. However, excessive Se in soil is toxic to plants and inhibits plant growth [18]. In this study, the biomass of the wood, bark, and leaves of the Xinfeng walnut increased in the Se20Cd0 treatment, but reduced in the Se200Cd0 treatment. This indicates that 20 μM Se may be an appropriate dosage for Xinfeng walnut growth, while 200 μM Se is excessive. Previous studies show that excessive Cd could lead to stunted plant growth, chlorosis, and even cell death [1,40]. In this study, Cd addition decreased the biomass of Xinfeng walnut. This indicates that 5 mM of Cd could inhibit walnut growth. However, after the application of Se, the Cd-induced inhibition on roots and leaves was alleviated (Table 2). This indicates that Se application (Se20 and Se200) is beneficial for the growth of walnuts with Cd exposure.

In previous studies, plants with leaf Cd concentration higher than 100 μg g^{-1} dry weight were defined as hyperaccumulating plants [1,41]. In some herbaceous Cd hyperaccumulators, Cd concentration was as high as 3000 μg g^{-1} dry weight [42]. Several studies found that some woody plants, such as *Populus* and *Salix* species, can accumulate considerable levels of Cd in their aerial parts [43,44]. The Cd concentration reached 116 μg g^{-1} dry biomass in the leaves of *Salix caprea* [45]. In some poplar species, Cd concentrations in root, bark, and leaves reached 1000, 300, and 100 μg g^{-1} dry weight, respectively [35,37]. In this study, Cd concentrations in the bark and root reached 2022 and 439 μg g^{-1} dry biomass, respectively, which were higher than those in poplar bark and root, respectively, in previous studies [37,46]. This may be related to differences in plant species, Cd concentration, and treatment duration.

Cd in soil could be absorbed by plant roots and translocated to the aerial organs via xylem [47]. Previous studies show that exogenous application of Se could reduce the accumulation of Cd in cucumber and rice [24,25]. In this study, application of 20 μM Se reduced the Cd concentration in root and wood, and application of 200 μM Se reduced Cd concentration in root, wood, bark, and leaves. Similarly, in *Helianthus annuus*, the

application of 5 and 10 μM Se decreased the Cd accumulation in roots and leaves, while the application of 20 μM Se did not alter the Cd concentration in roots and leaves [48]. This indicates that the application of Se could reduce Cd accumulation in walnuts, but the impact of Se on Cd accumulation varies with the change in Se concentration.

Cd may hinder nutrient uptake by plants, resulting in an imbalance of elements [49]. This study found that Cd addition enhanced the accumulation of Se in walnut wood treated with 20 μM Se, and the Se concentration in roots, wood, bark, and leaves of walnuts treated with 200 μM Se (Figure 1). Previous studies show that the application of inorganic Se fertilizers could effectively increase Se concentration in crops [18,50]. In this study, application of 20 μM Se increased Se concentration in walnut bark and leaves, and application of 200 μM Se increased Se concentration in walnut root, wood, bark, and leaves compared with non-Se treatment. This suggests that the Se sprayed on walnut leaves may preferentially accumulate in leaves and bark before being transported to wood and root.

4.2. Se Application Enhanced Antioxidant Enzyme Activities and Alleviated Cd-Induced Oxidative Stress

Cd is known to stimulate the formation of free radicals, which disrupts the plant defense system, resulting in the excessive accumulation of $O_2^{\bullet -}$ and H_2O_2 [51]. The content of MDA is used to assess the oxidative stress of chemical pollutants, including heavy metals [51,52]. In this study, under Cd exposure condition, the application of exogenous Se reduced the contents of ROS and MDA in walnut roots and leaves. This indicates that the oxidative stress induced by Cd could be relieved by Se. To reduce oxidative damage, plants evolved an antioxidant defense system consisting of a verity of enzymes, such as POD, CAT, APX, and AAO [51,53]. POD plays an important role in scavenging ROS produced under oxidative stress [54], and CAT, APX, and AAO could convert H_2O_2 to H_2O and O_2 [51,55]. For example, Ding found that a reduced activity of CAT could result in the accumulation of ROS in poplars [33]. In this study, the activities of CAT, APX, and AAO in walnut roots and CAT in leaves decreased after Cd exposure. The decreased activities of antioxidant enzymes could lead to the accumulation of ROS and oxidative stress. Furthermore, in this study, the application of Se increased the activities of CAT, POD, and AAO in root and CAT in the leaves of walnuts under Cd exposure. This suggests that Se could enhance the activities of antioxidant enzymes in walnut roots and leaves, thereby enhancing the ROS scavenging ability and reducing the Cd-induced oxidative stress.

In this study, exogenous application of 200 μM Se decreased the Cd accumulation in walnuts, which alleviated the growth inhibition and ROS accumulation induced by Cd. Furthermore, under Cd stress, the application of 20 μM Se increased the activities of CAT, POD, and AAO in walnut root and the activities of CAT and AAO in leaves, and the application of 200 μM Se enhanced the activities of CAT, POD, and AAO in walnut roots and CAT activity in leaves. Thus, Se application could alleviate the Cd-induced oxidative stress by increasing the ROS scavenging ability.

5. Conclusions

Exogenous application of Se could reduce the Cd concentration in the root, wood, bark, and leaves of Cd-stressed Xinfeng walnut, and the performance at the Se dose of 200 μM was better than that at the Se dose of 20μM. Furthermore, foliar application of 200 μM Se could remarkably increase Se concentration in walnuts compared with foliar application of 20 μM Se. Spraying Se on Cd-stressed walnuts could effectively reduce the growth inhibition induced by Cd. In addition, foliar-sprayed Se can be transported into the edible part of walnuts. The determination of appropriate Se dose will provide a practical guidance for the planting of Se-enriched walnuts. It should be noted that for walnuts grown in non-Cd-contaminated soils, the application rate of 200 μM Se may be excessive, and it is necessary to further explore the appropriate dose of Se.

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