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Selenium Treatment Alleviated Oxidative Alteration Generated by Cadmium in Sunflower Roots

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Authors' contributions

This work was carried out in collaboration between all authors. Author SI designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors DW and CY managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

The present study investigated the role of selenium (Se) in regulating cadmium (Cd)-induced oxidative stress in sunflower (*Helianthus annuus*) roots. Short-term exposure of plants to 20 µM Cd intensively increased hydrogen peroxides (H_2O_2), protein carbonyl (PCO) content and lipid peroxidation as indicated by malondialdehyde (MDA) accumulation. In contrast, Se (5, 10 and 20 µM) pretreatment alleviated the oxidative damages as evidenced by the lowered H_2O_2, protein carbonyl (PCO) and MDA content. Concomitantly, Se treatment enhanced the activities of catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione peroxidase (GPX, EC 1.11.1.9), but lowered that of superoxide dismutase (SOD, EC 1.15.1.1) in the root exposed Cd. Taken together, our results strongly suggest that exogenous Se may improve the tolerance of the plant to the Cd-induced oxidative stress.

Keywords: Cadmium; oxidative damages; Helianthus annuus; selenium antioxidants.

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1. INTRODUCTION

Cadmium (Cd) is a strong environmental pollutant with high toxicity to animals and plants. It is easily taken up by roots and transported to other parts of the plant, being toxic to living cells at very low concentrations. When taken up in excess by plants, it induces various visible symptoms of phytotoxicity, e.g. chlorosis, plant growth reduction, browning of root tips, and eventually death [1]. Cadmium phytotoxicity may result from alterations of various physiological processes caused at cellular level by inactivating enzymes, blocking functional groups of important molecules and disrupting membrane integrity. A rather common consequence of Cd toxicity is the enhanced production of reactive oxygen species (ROS) due to interference with electron transport activities, especially that of chloroplast membranes [2]. This increase in ROS exposes cells to oxidative stress leading to lipid peroxidation, biological macromolecule alteration, membrane dismantling, ion leakage, and DNA damage [3].

The principal mechanisms of plant response to Cd stress include phytochelatin-based sequestration and compartmentalization processes, as well as additional defense mechanisms, based on cell wall immobilization, plasma membrane exclusion, induction of stress proteins, etc. The degree to which higher plants are able to take up Cd depends on its concentration in the soil and its bioavailability, modulated by the presence of organic matter, pH, redox potential, temperature and concentrations of other elements. In particular, the uptake of Cd ions seems to be in competition for the same carrier with nutrients, such as potassium, calcium, magnesium, iron, manganese, cooper, and zinc [4].

Selenium (Se) is an essential micronutrient needed in antioxidation and hormone balance in human and animal cells. The antioxidant and anticarcinogenic properties attributed to some seleno compounds justify the increasing interest in growing selenium-enriched vegetables, which represent an important source of this element in the human diet [5].

According to current thinking, higher plants do not require Se, and the question of whether Se is an essential element to plants remains controversial. Recently, Se has been found to counteract the detrimental effects of diverse environmental stress, such as heavy metals [6], drought [7], salt [8] and senescence [9].

Many reports have shown that at lower doses Se can protect plants against the damage induced by heavy metals, including Pb, Cd, Zn, Al and Cu [5,6,9,10]. Whereas at high dose, Se acts as a pro-oxidant and cause damage to plants [11]. The relevant Cd-detoxification mechanisms by Se might be related to the inhibition of uptake and translocation of Cd from the roots to aboveground, increase of antioxidative ability, reduction of ROS and lipid peroxidation [12]. In the recent years, Se has been recognized for its potential antioxidant property and ability to increase the glutathione content in plant cell [8]. Therefore, the present study was undertaken to investigate the possible mediatory role of Se in protecting plants from Cd-induced oxidative stress.

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

Seeds of sunflower (*Helianthus annuus*) were sterilized and divided into two groups. One half of the seeds were soaked in Se (5, 10 and 20 µM) as selenate solution (Na_2SeO_4 10 H_2O) for 24 h, the other half of the seeds was soaked in water (control), and then the both groups were allowed to germinate on moist filter paper in the dark. Four-day-old, dark grown seedlings, were transferred to plastic beakers (6 L capacity, 6 plants per beaker) filled with nutrient solution containing: 1.0 mM $MgSO_4$, 2.5 mM $Ca(NO_3)_2$, 1.0 mM KH_2PO_4, 2.0 mM KNO_3, 2.0 mM NH_4Cl, 50 µM EDTA–Fe–K, 30 µM H_3BO_3, 10 µM $MnSO_4$, 1.0 µM $ZnSO_4$, 1.0 µM $CuSO_4$ and 30 µM $(NH4)_6Mo_7O_{24}$. After an initial growth period of 5 days, treatments were performed by adding 20 µM $CdCl_2$ to the nutrient solution. Cd dose used in this work are chosen appropriately to expose the plants from moderate levels of Cd. Plants were grown in a growth chamber with a 16-h-day (25ºC)/8-h-night (20ºC) cycle, an irradiance of 150 µmol m^{-2} s^{-1}, and 65-75% relative humidity. The nutrient solution was buffered to pH 5.5 with HCl/KOH, aerated, and changed twice per week. After 4 days of Cd-treatment, all plant organs were harvested, thoroughly washed with water, soaked in 20 mM EDTA for 15 min to remove adsorbed metals on the root surfaces and rinsed with distilled water. For biochemical analyses, sunflower roots were harvested and immediately stored in nitrogen

liquid. In each treatment group, five plants were examined for biochemical analysis.

2.2 Determination of Ion Concentrations

Dry plant material was powdered and wet-digested in acid mixture (HNO_3:$HClO_4$, 3:1, v/v) at 100ºC. Ion concentrations were estimated by atomic absorption spectrophotometry (Perkin-Elmer, Analyst 300) using an air–acetylene flame.

2.3 Determination of Lipid Peroxidation, Hydrogen Peroxide, Membrane Permeability and Protein Carbonyl Contents

The level of lipid peroxidation in plant leaves was determined by estimation of the thiobarbituric acid (TBA) reactive substances which was expressed as the malondialdehyde (MDA) concentration based on the method of Hodges et al. [13]. Lipid peroxidation level was expressed as nmol MDA formed using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Hydrogen peroxide (H_2O_2) levels were determined according to Sergiev et al. [14]. Results were expressed as nmol g^{-1} FM.

Membrane permeability was determined as follows. Fresh root sample (100 mg) was stirred for 30 min in deionised water followed by measurement of bathing medium conductivity (EC1). Then, the sample was boiled for 15 min and the final conductivity (EC2) of the medium was measured. Electrolyte leakage (%) was calculated using the formula: (EC1/EC2) x 100.

Protein carbonyls were determined using 2,4-dinitrophenylhydrazine (DNPH) and the basis of the assay involved the reaction between protein carbonyl and DNPH to form protein hydrazone [15]. The absorbance was measured at 370 nm, using the molar extinction coefficient of DNPH, ε = 22,000 M^{-1} cm^{-1} and the results were expressed as nmol DNPH conjugated mg^{-1} protein.

2.4 Determination of Antioxidative Enzyme Activities

Frozen root tissue (0.4 g) was homogenized in 4 mL ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0, 0.4% PVPP) using a pre-chilled mortar and pestle. The homogenate was squeezed through a nylon mesh and centrifuged for 30 min at 14.000 *g* at 4ºC. The supernatant was used for assays of the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). All spectrophotometric analyses were conducted at 25ºC.

The activity of SOD (EC1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) following the method of Beauchamp and Fridovich [16]. SOD activity was expressed as U mg^{-1} protein.

CAT (EC1.11.1.6) activity was assayed by the decomposition of hydrogen peroxide according to Aebi [17]. CAT activity was expressed as µmol H_2O_2 min^{-1} mg^{-1} protein.

APX (EC1.11.1.1) activity was determined by the method of Nakano and Asada [18]. Activity of APX was calculated by using the molar extinction coefficient for ascorbate (ε=2.8 mM^{-1} cm^{-1}). Activity was expressed as U mg^{-1} protein.

GPX (EC 1.11.1.9) activity was measured by a spectrophotometric method according to Drotar et al. [19]. The enzyme activity was expressed as U mg^{-1} protein.

2.5 Determination of Soluble Protein Concentration

Soluble protein concentration was measured according to Bradford [20] using the bovine serum albumin (BSA) protein assay reagent (Pierce, BSA Protein Assay Kit, USA) with BSA as the standard protein. All spectrophotometric measurements were performed by using a Perkin Elmer's LAMBDA 25/35/45 UV/Vis spectrophotometer.

2.6 Statistical Analysis

The experimental design was randomized with eight treatments and five replicates per treatment. The experiment was repeated three times under the same conditions. All statistical analyses were carried out with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA). Significant differences between treatment effects were determined by 1-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons with statistical significance of $P<0.05$.

3. RESULTS

3.1 Effects of Se Pretreatment on Growth Response and Cadmium Toxicity

In the present study, we have examined the effect of Cd on developing sunflower roots after 4 days treatment of hydroponic culture, and the subsequent effect of Se in conferring tolerance to Cd in roots. Results presented in Fig. (1A,B) showed that cadmium (20 µM) application reduced root length and root fresh matter by about 41% and 69% in Cd-treated plants as compared to control. Se pretreatment enhanced root growth and the most prominent effect was observed at 5 µM Se (Fig. 1A, B).

Cadmium addition to the nutrient solution resulted in a high accumulation of this metal within roots reaching a value of 3.2 mg g^{-1} DW (Table 1). Se at all applied concentrations significantly decreased Cd uptake and the major effect was detected at 5 µM Se, the concentration that significantly decreased Cd accumulation by about 38% as compared to Cd-treated plants.

3.2 Effects of Se Pretreatment on Lipid Peroxidation, Hydrogen Peroxide, Membrane Permeability and Protein Carbonyl Contents in Cd-treated Roots

A major effect of Cd toxicity is the chemical modification of membrane lipids triggered by the oxidative stress. Damages to the root membranes were investigated by monitoring MDA content. Relative to control, lipid peroxidation rate increased upon Cd exposure by nearly 3 times (Table 2). By contrast, presoaking seeds with Se before Cd treatment decreased lipid peroxidation content. The ameliorating effect of Se was more marked in plant pretreated with low (5 µM) Se concentration (Table. 2).

As applied separately, Cd induced a significant increase in H_2O_2 content in root seedling by approximately 2 times as compared to the control (Table 2). Pretreatment with 5 and 10 µM Se before exposure to Cd decreased H_2O_2 concentration by respectively 32% and 27 % in comparison with plants subjected to Cd stress without Se application (Table 2).

The extent of membrane damage was assessed by determining the amount of electrolyte leakage (EL) from root cells. As compared to the control, Cd-treated roots exhibited a higher rate of EL (Table 2). The conductivity measurements showed that at low concentrations of Se the EL tended to decrease notably at 5 µM Se (EL decreased significantly by about 33% compared to Cd-treated roots) (Table 2).

The inhibitory effects of Se on protein carbonyl (PCO) content in Cd-treated roots are presented in Table 2. Compared to control, treatment of plant with 20 µM Cd increased PCO level in sunflower roots by approximately 42%. By contrast, pretreatment with Se before Cd application decreased the level of PCO by about 34% and 30% at 5 and 10 µM, respectively. In plants pretreated with Se and non-subjected to Cd stress, no significant difference with the control was detected in root MDA, H_2O_2, EL, and PCO concentrations.

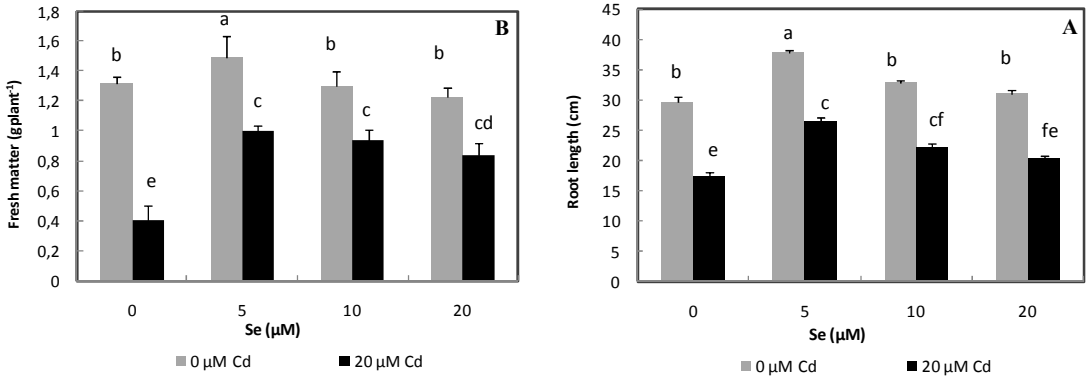

Fig. 1. Effects of Se pretreatment on root length (A) and root fresh matter (B) in sunflower seedling submitted during 4 days to 20 µM $CdCl_2$. Means of n=5±SE from three independent experiments. Different letters mean significance of difference between the treatments (P<0.05, Tukey's test)

Table 1. Effects of Se pretreatment on Cd concentrations (mg g^{-1}DM) in sunflower roots submitted during 4 days to 20 µM $CdCl_2$. Means of n=5±SE from three independent experiments. Different letters mean significance of difference between the treatments (P<0.05, Tukey's test)

Se (µM)	Cd concentration (mg g^{-1} DM)
	Roots
0	3.20±0.04a
5	1.98±0.02c
10	2.29± 0.01bc
20	2.52±0.06b

3.3 Effects of Se Pretreatment on Antioxidant Enzyme Activities in Cd-treated Roots

The changes in enzymatic antioxidants are shown in Fig. 2. The SOD activities in roots exposed to Cd treatment were observed to be about 38% higher than those of the control (Fig. 2A). By contrast, CAT, APX and GPX activities were decreased in Cd treated roots by about 40%, 38% and 42% as compared to control (Fig. 2B-D). Pre-soaking the seeds with Se upon Cd exposure resulted in a significant decrease in SOD activities and alleviated the inhibitory effect of Cd on CAT, APX and GPX activities. The most prominent effect was at 5 µM Se, the concentration that increase CAT, APX and GPX activities respectively by 26%, 23% and 30% relative to Cd-stressed plants (Fig. 2B-D).

4. DISCUSSION

The present study was performed to investigate the effect of Se in regulating Cd induced oxidative stress in sunflower roots. Cd treatment caused drastic reduction of sunflower roots, which could be due to the inhibition in cell division and elongation rate of cells that mainly occur by an irreversible inhibition of proton pump responsible for the process [21].

Se pretreatment enhanced root growth under Cd application and the most prominent effect was observed at lower dose of Se. Where as, at high dose Se acts as a pro-oxidant and cause various damage to plants [11]. Ameliorative impact of lower Se dose on sunflower roots was ascribed to the role of Se in nutrient uptake [22], stomatal regulation [23], cell membrane permeability and photosynthetic capacity [24]. As shown previously, Se ions are co-transported with Cd by the same protein carriers and bound to thiol groups of cysteine, an amino acid present in several proteins. Thus, competition for the specific binding sites in proteins can explains in part the protective effect of Se against Cd toxicity. Se-induced growth stimulation had also been noticed in other plant species [25], as well as the effect in suppressing the effects of biotic and abiotic stresses [5].

ROS and MDA are the well known markers for determining the extent of oxidative stress and considered to be the most important contributors for growth inhibition. Our results indicated that Cd exposed plants had enhanced levels of H_2O_2 and MDA which together affected the cell membrane functionality and integrity. These were in turn interfering with the biosynthesis of photosynthetic machinery and impaired the subsequent growth [26].

One of the defense mechanisms that protect plant from oxidative damage is the dismutation of superoxide anion radical to hydrogen peroxide by SOD which is further converted to water by GPX, using GSH as a substrate [27]. GSH is implicated directly in the reduction of most ROS [28] or indirectly by regenerating other potential water-soluble antioxidants, such as ascorbate (AsA), via the AsA–GSH cycle [29]. In the present study, the increased level of GSH in Cd-treated roots grown with Se application can be attributed to Se boosting the GSH synthesis. This result was supported by Hasanuzzaman et al. [8], who reported that Se accelerated efficient recycling of GSH depends on the glutathione reductase (GR) activity.

As a powerful scavenger of H_2O_2 and lipid hydroperoxides, GPX is widely and robustly activated by Se in diverse plants exposed to environmental stress [8,7,6]. As shown in our data, enhanced GPX activity induced by Se application in Cd-treated roots might conceal an expected increase in GSH levels. Consequently, this enhanced defense mechanism shifts the conversion of hydrogen peroxide to water thereby reducing the formation of highly reactive hydroxyl radical, and eventually leading to a lipid peroxidation decrease and hydrogen peroxide elimination. The hypothesis of Hartikainen et al. [11] that the increase in GPX resulted in reduced formation of H_2O_2 and lipid hydroperoxides is confirmed in the present study by recording a lower value of hydrogen peroxide production in Se treated plants coupled with increased CAT and reduced SOD activity.

Table 2. Effects of Se pretreatment on MDA, H_2O_2, EL and PCO in sunflower roots submitted during 4 days to 20 µM $CdCl_2$. Means of n=5±SE from three independent experiments. Different letters mean significance of difference between the treatments (P<0.05, Tukey's test). Data are expressed as: MDA (nmol g^{-1}FM), H_2O_2 (nmol g^{-1}FM), Electrolyte leakage: EL (%), PCO (nmol DNPH conjugated/mg protein)

Treatment		MDA	H_2O_2	EL	PCO
Se (µM)	Cd (µM)				
0	0	45.23±0.03d	31.41±0.09d	22.24±0.06d	11.58±0.10d
5	0	42.13±0.01d	33.48±0.01d	21.40±0.06d	10.29±0.07d
10	0	47.24±0.21d	31.57±0.02d	23.27±0.05d	11.06±0.08d
20	0	49.54±0.06d	35.99±0.05d	25.56±0.01d	11.75±0.08d
0	20	132.54±0.02a	63.15±0.10a	55.30±0.07a	19.94±0.08a
5	20	76.38±0.05c	42.83±0.10c	37.10±0.09c	13.04±0.07c
10	20	88.22±0.76c	46.07±0.10c	39.60±0.15c	13.89±0.05c
20	20	98.78±0.07b	52.35±0.10bc	44.68±0.12bc	15.19±0.08b

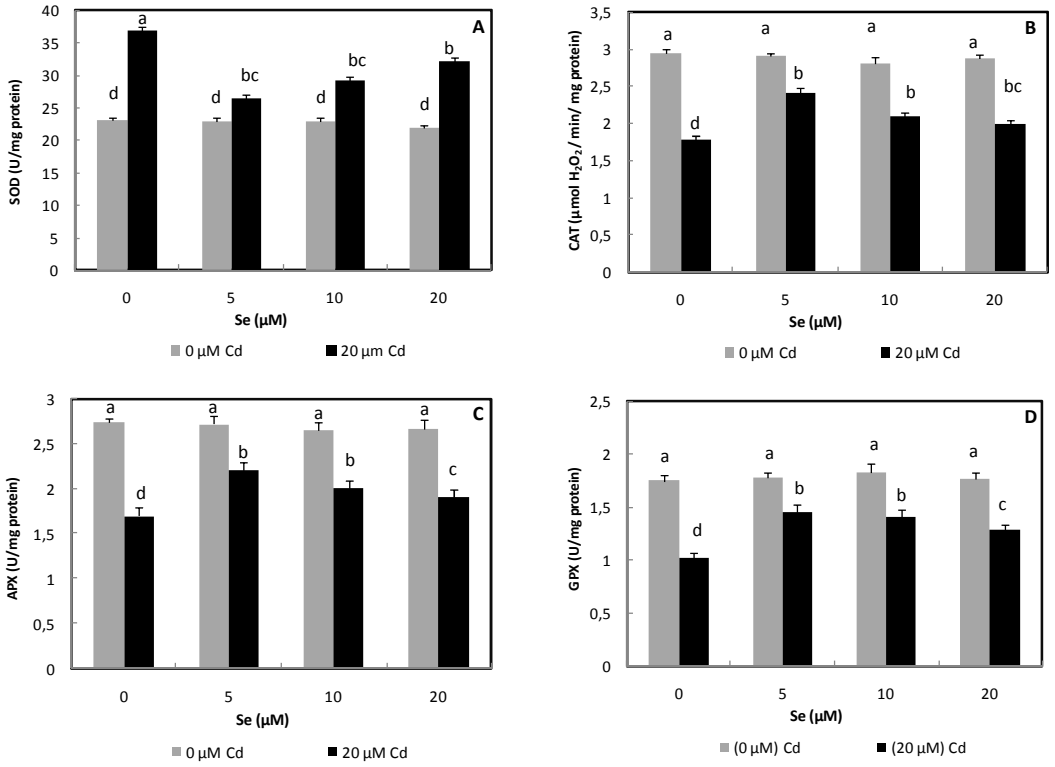

Fig. 2. Effects of Se pretreatment on SOD (A), CAT (B), APX (C) and GPX (D) activities in sunflower roots submitted during 4 days to 20 µM CdCl2. Means of n=5±SE from three independent experiments. Different letters mean significance of difference between the treatments (P<0.05, Tukey's test)

As an indispensible strategy to avoid damages induced by ROS, plants develop non-enzymatic and enzymatic antioxidant defense systems to protect plant cells from oxidative damage by scavenging ROS [6]. Our study showed that Cd treatments were found to have opposite effects on certain antioxidative enzymes. In sunflower roots, Cd induced elevated SOD, but decreased CAT, APX, and GPX activities. By contrast, Se at lower concentrations, led to higher activity of CAT, APX and GPX and concomitantly decreased SOD activity, thereby protecting the plants from oxidative damage as attested by the lower levels of leaf H_2O_2 and lipid peroxidation production. Se-induced variation in the activity of

oxidoreductase enzymes have been detected in many plant species [5].

Under stress conditions, ROS generation often exceeds the overall cellular antioxidative potential, which can cause oxidative damage of different cellular components such as proteins and nucleic acids [30]. Our results showed that Cd application increases PCO content in sunflower roots. Protein carbonylation can be ascribed to direct oxidation of amino acid side chains by ROS and/or by protein reactions with lipid peroxidation products, such as 4-hydroxy-2-nonenal. Our results corroborated with previous findings which demonstrated that Cd exposure enhanced oxidative species and proteins oxidation products [28]. On the other hand, Se-decreased lipid peroxidation may partially contribute to attenuate Cd alteration effect on protein content in sunflower leaves.

5. CONCLUSION

The present study illustrates the antioxidant properties of Se to avoid Cd toxicity in sunflower seedling. Therefore, based on these findings, Se might be able to down regulate Cd-induced oxidative damages through the inhibition of ROS production and indirectly by regulation of antioxidative system including GSH biosynthesis. The regulation of ROS levels by Se may be a key mechanism for counteracting environmental stress in plants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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