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Cadmium-induced oxidative damage and protective effects of *N*-acetyl-L-cysteine against cadmium toxicity in *Solanum nigrum* L.

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ABSTRACT

The effects of cadmium (Cd) on the accumulation of hydrogen peroxide (H_2O_2) and antioxidant enzyme activities in roots of *Solanum nigrum* L. and the role of *N*-acetyl-L-cysteine (NAC) as a cysteine (Cys) donor against Cd toxicity were investigated. Cd at 50 and 200 μM significantly increased the contents of thiobarbituric acid-reactive substances (TBARS), the production of H_2O_2 and superoxide anion ($O_2^{\bullet-}$), and the activities of catalase, guaiacol peroxidase, ascorbate peroxidase, glutathione peroxidase (GSH-Px), glutathione reductase, and superoxide dismutase. Experiments with diphenylene iodonium as an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and NaN_3 as an inhibitor of peroxidase showed that the major source of Cd-induced reactive oxygen species in the roots may include plasma membrane-bound NADPH oxidase and peroxidase. In addition, the effects of NAC on plant growth, antioxidant enzyme activity, and non-protein thiol content were analyzed. Under Cd stress, the addition of 500 μM NAC decreased the contents of TBARS and production of H_2O_2 and $O_2^{\bullet-}$, but increased levels of Cys and reduced glutathione (GSH), phytochelatins, and activity of GSH-Px in roots. These results suggest that NAC could protect plants from oxidative stress damage, and this protection seems to be performed via increased GSH biosynthesis. Furthermore, NAC treatment also increased the contents of protein thiols in *S. nigrum* roots. By using size-exclusion chromatography, we found involvement of NAC in the Cd tolerance mechanism through increased biosynthesis of Cd-binding proteins.

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1. Introduction

Cadmium (Cd) is one of the most toxic environmental pollutants for humans, animals, and plants, entering the environment mainly through mining operations, smelting of metals, alloy preparation, electroplating, municipal wastes, and phosphate fertilizers. Although Cd is a non-essential element, it is readily absorbed by plant roots and is then transported from roots to aerial parts [1]. Excess Cd can directly or indirectly inhibit biochemical and physiological processes of plants, such as photosynthesis, respiration, nitrogen and protein metabolism, nutrient uptake, and plant–water relationships, resulting in poor growth and low biomass [2].

Cd is a non-redox metal unable to participate in Fenton-type reactions, but it can promote reactive oxygen species (ROS) production and cause oxidative stress in plants [3–5]. ROS molecules are highly toxic and can oxidize biological macromolecules such as lipids, proteins and nucleic acids, thus causing lipid peroxidation, membrane damage, and inactivation of enzymes. To scavenge ROS and alleviate their deleterious effects, plants have developed protective enzymatic and non-enzymatic mechanisms. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR), whereas non-enzymatic antioxidants include glutathione and ascorbate [6]. These defense mechanisms against oxidative damage have been explicitly observed in plants subjected to Cd stress [5,7–11]. The antioxidative system response to heavy metals greatly depends on the plant species, age, and culture conditions [3].

In addition, plants exposed to Cd produce phytochelatins (PCs), metal-binding Cys-rich peptides with the general structure (γ-Glu-Cys)$_n$-Gly (n = 2–11) that may be involved in either homeostasis or detoxification of metal ions in plant cells [1]. PCs are synthesized non-translationally by the enzyme PC synthase, which is activated by metal ions such as Cd, Pb, Zn, and Cu, and uses reduced glutathione (GSH) as a substrate. Metals are bound in the cytosol, and the PC–metal complex is sequestrated in the vacuole, thereby reducing the concentration of free metal ions in the cytosol. The increased capacity of PC and GSH synthesis enhanced Cd tolerance and accumulation in Indian mustard [12]. GSH (γ-glutamyl-cysteinyl–glycine) is an abundant tripeptide of great importance in the living cell. A possible consequence of

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Cd-induced GSH synthesis would be the detoxification of Cd concentrations through the increased synthesis of PCs [13]. Apart from metal chelation, GSH is involved in multiple physiological functions such as intracellular redox state regulation, scavenging of ROS and transport of GSH-conjugated amino acids [14]. The level of GSH depends upon the availability of the substrate cysteine. Metallothioneins (MTs), a class of low-molecular-weight (6–7 kDa), Cys-rich proteins, may protect cells against the toxic effects of metals by chelating them via their Cys thiol groups. Unlike PCs, MTs are direct gene products. MT gene expression can be induced by certain metal ions as well as by a variety of physiological and chemical stressors [15]. Although it is widely accepted that MTs are primarily involved in cellular Cu and Zn homeostasis, some studies have reported that plant MTs mediate tolerance to Cd [16,17].

Recently, it has been reported that application of *N*-acetyl-L-cysteine (NAC, $C_5H_9NO_3S$) decreased the oxidative stress caused by herbicides [18] and UV-B [19]. NAC is a general antioxidant and can increase cellular pools of free radical scavengers. It is a synthetic derivative compound which acetylized from cysteine and acts as a GSH precursor by raising the intracellular concentration of cysteine, in turn metabolized to GSH [20]. Currently, limited information is available about the protective effects of NAC treatment on plant injuries caused by heavy metals.

Solanum nigrum L. is an annual weed that grows worldwide. Previous studies have shown that *S. nigrum* can accumulate large amounts of heavy metals, particularly Cd, in its aerial tissues [21,22]. Although some reports have examined the characteristics of Cd uptake by *S. nigrum* and its growth and physiological responses to Cd stress, such as antioxidative defenses and nitrogen metabolism [23,24], the mechanisms of metal tolerance and accumulation in *S. nigrum* remain unclear.

The primary objectives of this study were (1) to investigate effects of Cd on the production of ROS in roots of *S. nigrum*; (2) to reveal possible relationships between the toxic effects of Cd and changes in the activities of several enzymes involved in the antioxidant defense system; and (3) to examine whether NAC can protect *S. nigrum* from Cd toxicity, and if so, whether this effect is related to GSH content.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of *S. nigrum* were collected in close proximity to a smelting factory in the Xiangxi area (109°10′00″–109°22′30″E, 27°44′30″–29°28′00″N) of Hunan Province, China. After sterilization with 5% sodium hypochlorite solution for 15 min, the seeds were allowed to germinate at room temperature (20–25 °C) in plastic dishes filled with vermiculite. Approximately 3-week-old seedlings were transferred to vessels containing 2 l of Hoagland's nutrient solution. The pH of the nutrient solution was adjusted to 5.8 with NaOH or HCl, and the nutrient solution renewed every 2 days. Plants grew under controlled greenhouse conditions with natural light, 25/20 °C day/night temperatures, and relative humidity of 60–70%. Five-week-old seedlings were treated with different Cd or NAC concentrations in nutrient solution: 0 (control), 500 μM NAC, 50 μM Cd, 50 μM Cd + 500 μM NAC, 200 μM Cd, or 200 μM Cd + 500 μM NAC. The experiment was arranged using a randomized design with three replicate vessels each containing 18 seedlings. After treatment with Cd and NAC for 3 days, the root lengths were measured and the plants were harvested.

2.2. Treatments with enzyme inhibitors and ROS scavengers

The purpose of this experiment was to identify the origin and scavenging of ROS in plant roots under exposure to Cd. After treatment with 50 μM Cd for 3 days, the plant roots were immersed in 2 mM NAC, 1 mM ascorbic acid (AsA), 1 mM NaN_3 (as an inhibitor of POD), 100 units ml^{-1} SOD, 1 mM 3-amino-1,2,4-triazole (ATZ, as an inhibitor of CAT), 5 mM 4-aminophenol (PAP, as an inhibitor of APX), 2 mM mercaptosuccinic acid (MSA, as an inhibitor of glutathione peroxidase (GSH-Px)), 50 μM diphenylene iodonium (DPI, $C_{12}H_8ClI$), 1 mM *N-N*-diethyldithiocarbamate (DDC, as an inhibitor of SOD), or 10 mM Tiron (as a scavenger of $O_2^{\bullet-}$). DPI has frequently been used to inhibit ROS production mediated by flavoenzymes, particularly NADPH oxidase. After 1 h, the plant roots were harvest for the detection of H_2O_2 and $O_2^{\bullet-}$levels and for POD activity.

2.3. Determination of lipid peroxidation, H_2O_2, and $O_2^{\bullet-}$in the root extracts

Lipid peroxidation was measured by the content of total thiobarbituric acid ($C_4H_4N_2O_2S$)-reactive substance (TBARS), as described by Jiang and Zhang [25]. TBARS was considered as an index of the oxidation of polyunsaturated fatty acids. The content of H_2O_2 in the root extracts was measured by monitoring the absorbance at 390 nm of the mixtures following the method described by Loreto and Velikova [26]. The production rate of $O_2^{\bullet-}$was measured by monitoring the absorbance at 530 nm of the nitrite formation from hydroxylamine hydrochloride in the presence of $O_2^{\bullet-}$, as described by Jiang and Zhang [25].

2.4. Histochemical detection of ROS

Histochemical detection of H_2O_2 and $O_2^{\bullet-}$in plant root-tips was performed according to the method described by Romero-Puertas et al. [27]. For H_2O_2 detection, plant roots were immerged in a 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer solution (pH 6.5) containing 0.1% (w/v) 3, 3′-diaminobenzidine (DAB, $C_{12}H_{14}N_4 \cdot 4HCl \cdot xH_2O$) and then incubated at room temperature for 8 h in the dark. Deep brown polymerization product can be formed by the reaction of DAB with H_2O_2. The $O_2^{\bullet-}$formation was detected by incubating roots in 0.1% nitroblue tetrazolium (NBT) solution for 30 min under light conditions.

2.5. Assays of antioxidant enzyme activities

Soluble proteins in plant roots were extracted with 50 mM ice-cold potassium phosphate buffer (pH 7.0) containing 1% (w/v) polyvinylpyrrolidone (PVP) and 1 mM ethylene diamine tetraacetic acid (EDTA). The activities of CAT, APX, GR, and SOD were determined as described by Jiang and Zhang [25]. POD activity was determined according to the method described by Zheng and Van Huystee [28]. GSH-Px activity was determined according to Aravind and Prasad [29]. Protein content was estimated according to Bradford [30] using bovine serum albumin as a standard.

2.6. Determination of thiols, glutathione, cysteine, and PCs contents

The contents of total thiols were estimated according to Ellman [31]. Non-protein thiols (NPT) and total glutathione were determined following the method described by De Vos et al. [32]. The protein thiols (PT) were calculated by subtracting the non-protein thiols (NPT) from total thiols. Oxidized glutathione (GSSG) was measured in a similar manner as total glutathione, except that the supernatant was incubated with 2-vinylpyridine and triethanolamine. GSH was calculated by subtracting the amount of GSSG from the amount of total glutathione. Cysteine was measured by the ninhydrin method described by Oven et al. [33], and the absorbance of the mixture was read at 560 nm. PCs levels could be estimated from the difference between total NPT and GSH [34]. In

Table 1
Effects of NAC and Cd treatments on net root elongation, dry weight and Cd concentration in roots of *S. nigrum*.

Treatment (μM)	Net root elongation[a] (cm)		Root dry weight[b] (g)		Shoot dry weight (g)		Root Cd content (μg/g DW)		Shoot Cd content (μg/g DW)	
	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC
0 Cd	3.50	3.73	0.730	0.722	4.87	4.89				
50 Cd	1.97	3.00	0.684	0.707	4.70	4.80	123	121	32.3	28.3
200Cd	0.983	1.63	0.644	0.675	4.62	4.68	187	170	51.3	47.0
ANOVA F ratio										
NAC	63.8***		4.93*		10.0**		5.37*		12.38**	
Cd	278***		31.4**		59.4***		194***		261***	
NAC × Cd	8.34**		3.05NS		1.46NS		3.62NS		0.01NS	
Error d.f.	30		12		12		8		8	
LSD ($P < 0.05$)	0.283		0.0259		0.0661		13.1		3.81	

Plants were exposed to various treatments for 3 days. NS: not significant.
[a] Values of net root elongation are means of six different experiments.
[b] Values of dry weights represent 27 individuals of roots or shoots with three different experiments.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

this study, PCs levels were calculated from the difference between total NPTs and the summation of GSH and cysteine due to the addition of NAC.

2.7. Assay for Cd-binding complexes

Cd-binding complexes in roots of *S. nigrum* were investigated using size-exclusion chromatography. Fresh samples were homogenized in a buffer containing 50 mM Tris–HCl (pH 8.0), 1 mM ascorbic acid, 1 mM DTT, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) according to the method described by Malec et al. [35] with modifications. All steps were carried out at 4 °C. The supernatant was applied to a medium Sephadex G-50 column (1.6 cm × 130 cm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0). Cd-binding complexes were eluted using the same buffer with a flow rate of 0.5 ml min^{-1}. The eluted solution was collected in a 5-ml fraction, and the 254-nm absorbance and Cd concentrations were determined using a flame atomic absorption spectrometer (Jena Nov 400).

2.8. Determination of Cd concentrations in plant tissues

The plants were divided into roots and shoots and dried at 80 °C to a constant weight. The dried plant samples were ground into a fine powder with an agate mortar and completely digested with a solution of HNO_3 and $HClO_4$ (87:13, v/v). The Cd concentrations were determined using a flame atomic absorption spectrometer (Jena Nov 400).

2.9. Statistical analysis

Data were analyzed using SPSS ver. 13.0 (Statistical Package for Social Science for Windows, SPSS, Inc., Chicago, IL, USA). All values reported in this paper are means values of three replicates. Statistical analyses were carried out by analysis of variance (ANOVA) tests. Significant differences among mean values were determined by the LSD at $P < 0.05$.

3. Results

3.1. Plant growth and Cd accumulation

As compared with the control group, treatments with 50 and 200 μM Cd for 3 days significantly decreased net root elongation and root and shoot dry weights of *S. nigrum* (Table 1). The addition of 500 μM NAC improved the growth of plants grown under the Cd treatment. In the absence of Cd, however, NAC treatment did not have any significant effect on plant growth.

The Cd concentrations in the shoots and roots of *S. nigrum* were higher in the 200-μM Cd treatment than in the 50-μM Cd treatment (Table 1). The addition of 500 μM NAC significantly decreased the concentration of Cd in roots under the 200-μM Cd treatment. The shoots of *S. nigrum* accumulated less Cd than the roots did under all treatments.

Table 2
Effects of NAC and Cd treatments on contents of TBARS and H_2O_2 and production rate of $O_2^{\bullet -}$ in roots of *S. nigrum*.

Treatment (μM)	TBARS (μmol g^{-1} FW)		H_2O_2 (μmol g^{-1} FW)		$O_2^{\cdot -}$ (nmol min^{-1} g^{-1} FW)	
	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC
0 Cd	1.19	1.34	0.938	1.09	5.79	6.04
50 Cd	2.21	1.32	1.45	1.27	7.95	5.36
200Cd	2.54	2.13	1.70	1.48	10.0	8.78
ANOVA F ratio						
NAC	14.7**		2.51NS		10.6**	
Cd	37.9***		43.6***		33.4***	
NAC × Cd	8.75**		5.54*		4.99*	
Error d.f.	12		12		12	
LSD($P < 0.05$)	0.379		0.192		1.382	

Plants were exposed to various treatments for 3 days. NS: not significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

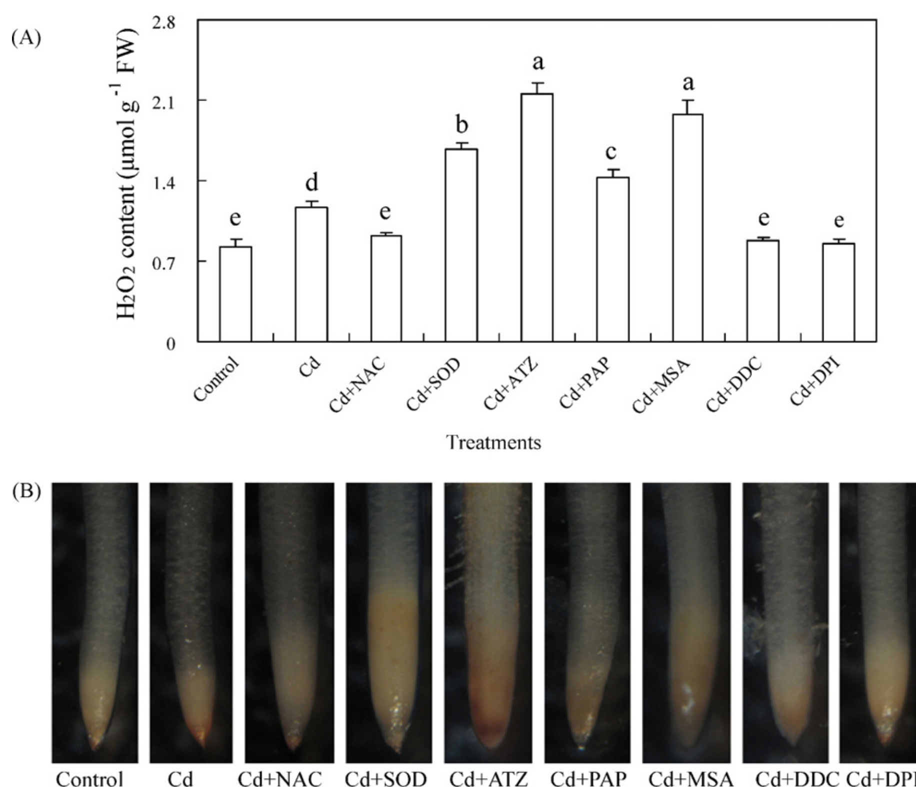

Fig. 1. Cd and additional treatments with NAC, SOD, ATZ, PAP, MSA, DDC, and DPI induced changes in H_2O_2 production in roots of *S. nigrum*. (A) The total contents of H_2O_2 in roots and (B) histochemical localization of H_2O_2 by DAB staining. Plants were exposed to 0 (control) or 50-μM Cd treatment for 3 days, and their roots were subsequently treated with 2 mM NAC, 100 units ml^{-1} SOD, 1 mM ATZ, 5 mM PAP, 2 mM MSA, 1 mM DDC, or 50 μM DPI. After 1 h, excised roots were homogenized, and the H_2O_2 content was assayed by spectrophotometry or immersed in DAB for histochemical detection of H_2O_2.

3.2. Lipid peroxidation and H_2O_2 and $O_2^{\bullet-}$ production

The level of lipid peroxidation products, determined by the TBARS content, was significantly higher in *S. nigrum* roots treated with 50 and 200 μM Cd than in those of the control group (Table 2). Production of H_2O_2 and $O_2^{\bullet-}$ was also higher in the Cd treatments than in the control. The addition of 500 μM NAC significantly decreased the TBARS content and H_2O_2 and $O_2^{\bullet-}$ production in the roots of *S. nigrum* grown under the Cd treatment (Table 2).

After 3 days of treatment with Cd, the roots of *S. nigrum* were treated with ROS generators, scavengers, or antioxidant enzymic inhibitors. Results showed that H_2O_2 accumulation in roots treated with 50 μM Cd decreased under treatment with 2 mM NAC, 50 μM DPI (an inhibitor of NADPH oxidase), or 1 mM DDC (an inhibitor of SOD), but increased under treatment with 1 mM ATZ (an inhibitor of CAT), 5 mM PAP (an inhibitor of APX), 2 mM MSA (an inhibitor of GSH-Px), or 100 units ml^{-1} SOD (Fig. 1). The Cd-induced accumulation of $O_2^{\bullet-}$ in roots could be increased by treatment with DDC and decreased by treatment with NAC, DPI, SOD, or Tiron ($O_2^{\bullet-}$ scavenger) (Fig. 2).

3.3. Activities of antioxidant enzymes

The activities of antioxidant enzymes (CAT, APX, SOD, POD, GR, and GSH-Px) in *S. nigrum* roots exhibited an increasing trend in response to Cd treatment (Table 3). Control plant roots possessed the lowest values, whereas NAC treatment caused a significant increase in the activities of CAT, APX, and GR in the roots of *S. nigrum* grown under the 200-μM Cd treatment. Maximum activities of CAT, APX, SOD, GR, and GSH-Px were recorded in the plants exposed to a combined treatment with 500 μM NAC and 200 μM Cd. GSH-Px showed a relatively significant difference in activity among different treatments when compared with CAT, APX, SOD, and GR. NAC treatment increased the activity of GSH-Px, but significantly decreased the activity of POD in the roots of *S. nigrum* grown under the Cd treatment.

After 3 days of treatment with Cd, the roots of *S. nigrum* were treated with 1 mM NaN_3 (an inhibitor of POD) for 1 h. Results show that NaN_3 treatment significantly decreased the activity of POD and production of H_2O_2 in the roots of *S. nigrum* (Fig. 3).

3.4. Protein thiol and non-protein thiol contents

The levels of protein thiols and non-protein thiols increased in the roots exposed to Cd treatment compared with those in the control group (Table 4). The addition of 500 μM NAC also significantly increased the levels of PT and NPT in the roots. The highest levels of *S. nigrum* PT and NPT were observed in plants that received 200 μM Cd together with 500 μM NAC. Values were 162% and 168% greater than that of the control, respectively.

The GSH, Cys, and PC contents in the roots of *S. nigrum* were significantly increased by NAC treatment in all levels of Cd treatment (Table 4). Cd treatment significantly increased the GSSG, GSH + GSSG, and PC contents. PC contents reached 197.5 and 260.4 nmol SH g^{-1} FW in the 200-μM Cd-treated plants without and with NAC, respectively, which were 5.2-fold and 6.8-fold that of the control groups (without Cd treatment). In contrast, increasing Cd supply did not have any significant effect on the GSH content. The presence of Cd in the medium decreased the GSH/GSSG ratio. The decrease was more pronounced in plants treated in the absence of NAC than in the presence of NAC. The addition of 500 μM NAC increased the GSH/GSSG ratio in the roots of *S. nigrum* exposed to Cd treatment.

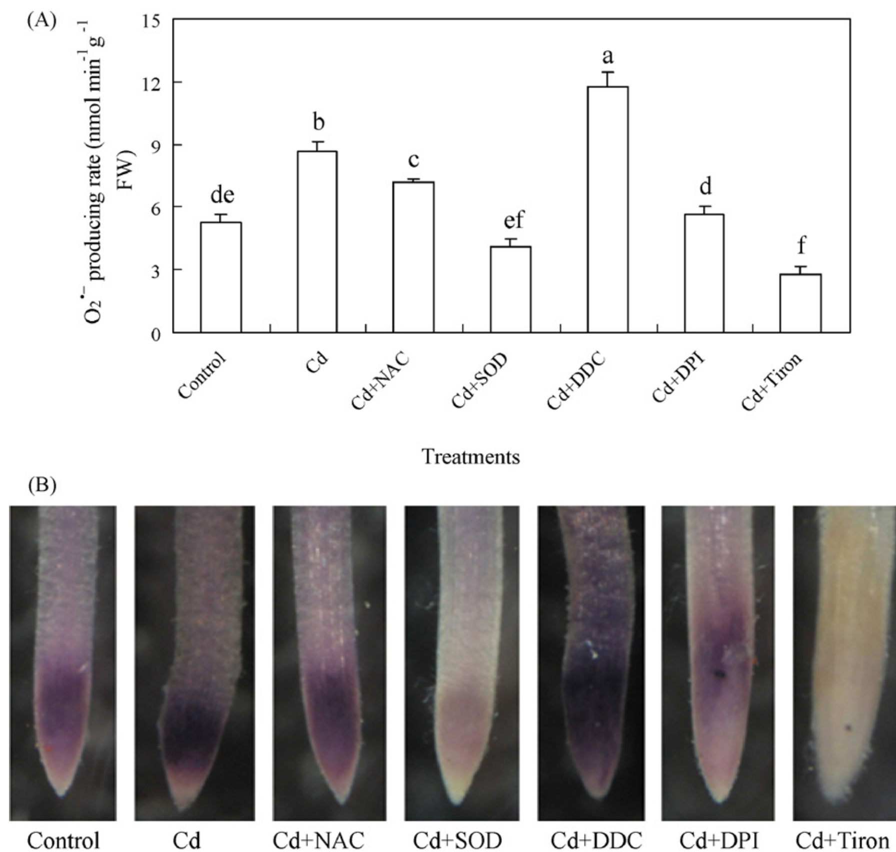

Fig. 2. Cd and additional treatments with NAC, SOD, DDC, DPI, and Tiron induced changes in $O_2^{\bullet-}$ production in roots of *S. nigrum*. (A) The production rate total contents of $O_2^{\bullet-}$ in roots and (B) histochemical localization of $O_2^{\bullet-}$ by NBT staining. Plants were exposed to 0 (control) or 50 μM Cd treatment for 3 days, and their roots were subsequently treated with 2 mM NAC, 100 units ml^{-1} SOD, 1 mM DDC, 50 μM DPI, or 10 mM Tiron. After 1 h, excised roots were homogenized, and the production rate of $O_2^{\bullet-}$ was assayed by spectrophotometry or immersed in NBT for histochemical detection of $O_2^{\bullet-}$.

3.5. Cd-binding complexes

The elution fraction of the root extracts and Cd contents in the extracts from the plants grown in different treatments of Cd and NAC are shown in Fig. 4. Generally, two UV-absorbing peaks were observed at volumes of 55–75 and 145–185 ml, respectively. The first UV-absorbing peak was far higher than the second one. Compared with the control group, the treatment with 50 μM Cd increased both UV-absorbing peaks. The content of Cd detected in the second peak was much higher than that in the first one. Approximately 3.2% and 82.2% of the total intracellular Cd in the roots, respectively, were bound to high- and low-molecular-weight complexes. In the presence of 50 μM Cd, the 500-μM NAC treatment also increased both UV-absorbing peaks and increased Cd content in the first peak. Cd content in the first peak increased 4.6-fold, in comparison with that in the treatment of 50 μM Cd alone.

4. Discussion

Oxidative stress is a well-documented effect of metal toxicity in plants [3]. Cd treatment caused an enhanced level of TBARS in *S.*

Table 3
Effects of NAC and Cd treatments on activities of CAT, APX, SOD, POD, GR and GSH-Px in roots of *S. nigrum*.

Treatment (μM)	CAT (μmol min^{-1} mg^{-1} protein)		APX (μmol min^{-1} mg^{-1} protein)		SOD (U mg^{-1} protein)		POD (μmol min^{-1} mg^{-1} protein)		GR (nmol min^{-1} mg^{-1} protein)		GSH-Px (μmol min^{-1} g^{-1} FW)	
	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC
0 Cd	28.1	26.9	7.75	8.62	0.979	0.972	93.9	102	4.03	4.38	28.8	44.5
50 Cd	37.0	29.3	8.18	8.84	1.14	1.21	126	109	5.52	5.12	37.8	49.3
200Cd	44.1	47.0	9.96	11.4	1.37	1.52	135	116	6.31	7.08	60.6	66.9
ANOVA F ratio												
NAC	18.4**		12.9**		1.81NS		8.95*		2.24NS		71.3***	
Cd	513***		32.0***		25.7***		29.3***		78.8***		152***	
NAC × Cd	43.8***		0.75NS		0.77NS		8.03**		4.41*		4.33*	
Error d.f.	12		12		12		12		12		12	
LSD (P < 0.05)	1.78		1.05		0.203		11.4		0.612		4.98	

Plants were exposed to various treatments for 3 days. NS: not significant.
* P < 0.05.
** P < 0.01.
*** P < 0.001.

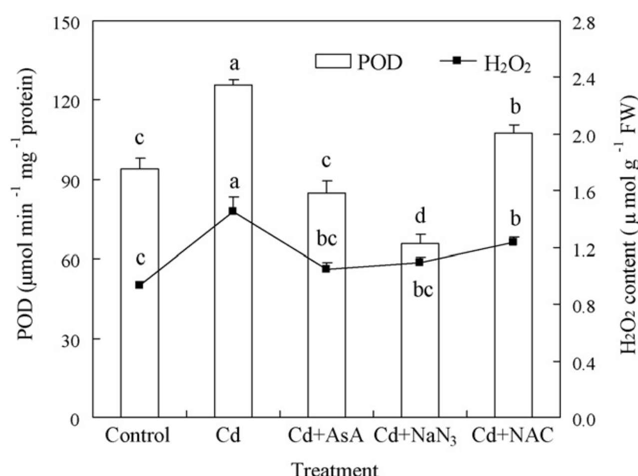

Fig. 3. Cd and additional treatments with NAC, AsA, or NaN_3 induced changes in POD activity and H_2O_2 production in roots of *S. nigrum*. Plants were exposed to 0 (control) or 50 μM Cd treatment for 3 days, and their roots were subsequently treated with 2 mM NAC, 1 mM AsA, or 1 mM NaN_3. After 1 h, excised roots were homogenized, and the H_2O_2 content and POD activity were assayed by spectrophotometry.

nigrum roots (Table 2), which is an index of lipid peroxidation and, therefore, of oxidative stress. Similarly, considerable H_2O_2 formation was observed in the Cd-treated roots of *S. nigrum* compared with the control. Accumulation of H_2O_2 has been observed also in other Cd-exposed plant species [27,36,37]. Under most conditions, H_2O_2 in plants can be efficiently scavenged by either CAT or the ascorbate–glutathione cycle, during which APX reduces H_2O_2 to H_2O. Efficient recycling of GSH is ensured by GR. Furthermore, GSH-Px catalyzes the reduction of H_2O_2 by GSH. SOD catalyses the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2. In this study, treatment with Cd for 3 days increased the activities of SOD, CAT, APX, GSH-Px, and GR in roots of *S. nigrum*, indicating that *S. nigrum* roots have the capacity to adapt to Cd toxicity by developing an antioxidant defense system. The increase in the activity of antioxidant enzymes, such as SOD, CAT, APX, and GR, after Cd exposure also has been observed in other plant species [5,7,10,11]. In contrast, Cd depressed the activities of CAT and POD in leaves of *Pisum sativum* [8] and activities of SOD, CAT, APX and GR in leaves of *Tagetes erecta* [9]. In the less Cd-sensitive pea genotypes, APX activity decreased, whereas the activities of CAT and POD significantly increased [38]. Improvement of stress tolerance is often related to an increase in activity of antioxidant enzymes [39].

Accumulation of H_2O_2 in plants could be due to enhanced H_2O_2 production and/or to decreased capacity for H_2O_2 scavenging. In Cd-treated *S. nigrum* roots, the former mechanism likely occurred, as we did not observe a decrease in the activities of the major H_2O_2-scavenging enzymes CAT, APX, and GSH-Px. H_2O_2 can be generated by specific enzymes such as SOD, NADPH oxidase, xanthine oxidase, amine oxidase, and a cell wall peroxidase [40]. Our results showed that DPI, a relatively specific inhibitor for NADPH oxidase [41], suppressed the production of both $O_2^{\bullet-}$ and H_2O_2 in the roots of Cd-exposed *S. nigrum* (Figs. 1 and 2). Furthermore, the inhibition of SOD by DDC increased the accumulation of $O_2^{\bullet-}$ and decreased the accumulation of H_2O_2. These results showed that Cd-induced H_2O_2 production in *S. nigrum* roots originated, at least in part, from plasma membrane (PM)-bound NADPH oxidases. PM-bound NADPH oxidases can use cytosolic NADPH to produce $O_2^{\bullet-}$, which quickly dismutates to H_2O_2 via SOD. The involvement of NADPH oxidase in H_2O_2 production was recently shown to be induced in other plants by Cd [4,5,42] and Cu [43–45]. In addition to NADPH oxidase, several other potential enzymatic sources of ROS exist [6]. PODs have been suggested to play an important role in the formation of H_2O_2. In this study, the total activity of POD, using guaiacol as a substrate, increased significantly in roots of Cd-treated *S. nigrum* compared to the control. The inhibition of POD by 1 mM NaN_3, a well-known POD and cytochrome oxidase inhibitor, decreased the accumulation of H_2O_2 in roots of *S. nigrum* treated with 50 μM Cd (Fig. 3). This result shows that POD activation is also associated with Cd-induced H_2O_2 production. Similar results have been reported for *Phaseolus aureus* and *Vicia sativa* [5]. In contrast, some authors have reported that PODs are not implicated in ROS production by Cd-treated tobacco BY-2 cells [4] and Cu-treated *Elsholtzia haichowensis* roots and leaves [44,46].

The addition of NAC decreased the level of lipid peroxidation and production of H_2O_2 and $O_2^{\bullet-}$, indicating that NAC can protect plants from oxidative stress damage and increase Cd tolerance in *S. nigrum* (Table 2). This protection seems to be performed via increased GSH biosynthesis, as higher levels of non-protein thiols (Cys, GSH, and PCs) and activity of GSH-Px were observed in the presence of NAC compared with in its absence. However, Ohlsson et al. [47] reported that NAC treatment did not influence metal tolerance in *Salix* despite effects on the GSH level. The activities of major H_2O_2-scavenging enzymes also indicated that protection of NAC from oxidative stress damage did not result from the activation of CAT and APX. A significant increase in CAT and APX activity by NAC treatment was only observed under the 200 μM Cd treatment (Table 3).

GSH protects cells from the oxidative stress induced by heavy metals and is the direct precursor of PCs. Cd treatment has been reported to induce a marked depletion of GSH levels due to PC

Table 4
Effects of Cd and NAC treatments on contents of protein thiols, non-protein thiols, total glutathione (reduced and oxidized), cysteine, and PCs in roots of *S. nigrum*.

Treatment (μM)	Protein thiols (nmol SH g^{-1} FW)		Non-protein thiols (nmol SH g^{-1} FW)		GSH (nmol g^{-1} FW)		GSSG (nmol g^{-1} FW)		GSH + GSSG (nmol g^{-1} FW)		Cysteine (nmol SH g^{-1} FW)		PCs (nmol SH g^{-1} FW)	
	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC	0 NAC	500 NAC
0 Cd	646.1	710.4	417.2	518.9	348.6	364.2	78.77	92.84	427.4	457.0	30.38	88.61	38.19	82.75
50 Cd	759.3	894.0	519.4	671.1	320.0	369.6	120.7	98.52	440.7	468.1	20.17	71.44	179.3	230.0
200 Cd	821.6	1048	522.8	703.9	313.8	385.7	134.3	125.4	448.1	511.1	17.06	58.94	197.5	260.4
ANOVA F ratio														
NAC	38.37***		541.6***		62.29***		3.080 NS		45.88***		481.8***		70.58***	
Cd	42.21***		226.9***		1.350NS		61.96***		13.91**		32.12***		352.2***	
NAC × Cd	4.180*		12.55**		8.000**		10.73**		3.790 NS		5.080*		4.050*	
Error d.f.	12		12		12		12		12		12		12	
LSD (P < 0.05)	86.32		23.20		21.84		12.21		22.29		8.606		22.00	

Plants were exposed to various treatments for 3 days. NS: not significant.
* P < 0.05.
** P < 0.01.
*** P < 0.001.

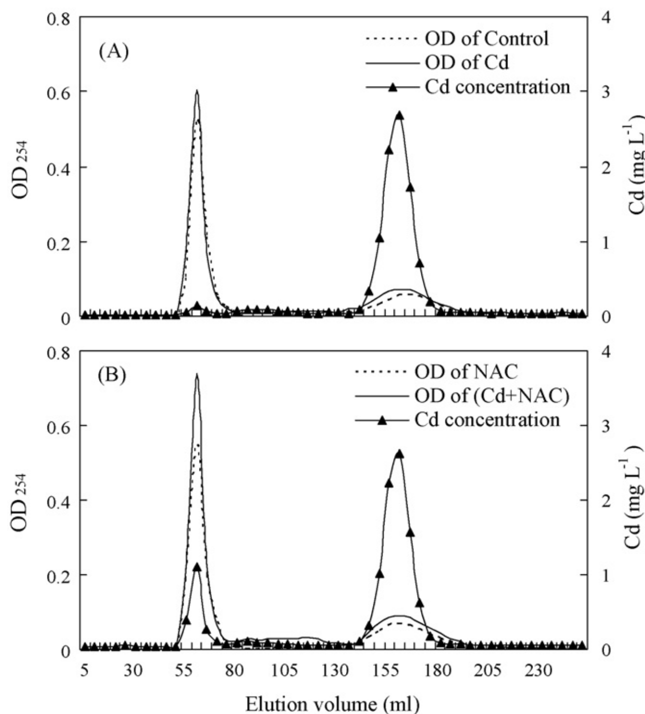

Fig. 4. Gel filtration of Cd-binding protein and Cd concentration in the roots of *S. nigrum*. Plants were treated with 0 (control) and 50 μM Cd in the absence of NAC (A) or in the presence of 500 μM NAC (B) for 3 days.

biosynthesis in different plants [12,48–50]. GSH depletion could cause oxidative stress [32]. Metwally et al. [38] reported that more Cd-sensitive pea genotypes had decreased root GSH concentrations, whereas less sensitive genotypes had increased root GSH concentrations in response to Cd treatment. GSH concentrations were higher in Cd-tolerant genotypes than in Cd-sensitive genotypes of *Silene vulgaris* [51]. Genetic studies have confirmed a GSH-deficient mutant of *Arabidopsis* that is hypersensitive to Cd [15]. In the present study, GSH concentrations decreased by 8.2% and 10.0% in the roots of *S. nigrum* plants treated with 50 and 200 μM Cd, respectively, compared with those in the control group in the absence of NAC (Table 4). In contrast, total non-protein thiol contents were significantly increased by Cd treatment. This increase in concentration of non-protein thiols is attributable to an increase in PCs biosynthesis. PCs are rapidly induced in cells and tissues exposed to a range of heavy metal ions, particularly Cd [52]. The biosynthesis of PCs could chelate heavy metal ions in PC–Cd complexes. But in the presence of NAC, concentrations of total non-protein thiols, GSH, and PCs increased significantly in the roots of *S. nigrum* exposed to Cd. These results suggested that GSH was continuously synthesized to meet the requirement for PC biosynthesis and other stress related processes. The elevated GSH level was due to deacylation and conversion of NAC to GSH.

It has been well established that not only GSH concentration, but also the GSH/GSSG ratio is important to maintain cellular redox status [53]. In the absence of NAC, significantly decreased concentrations of GSH coinciding with an increase in GSSG concentration were observed under both Cd concentrations. The GSH/GSSG ratio decreased by 40.0% at 50 μM Cd and by 47.2% at 200 μM Cd compared with the control. Although significantly higher GR activity was observed in roots of Cd-treated plants, this seemed insufficient to maintain the GSH redox balance at the same level as in the control. However, in the presence of NAC, an increasing trend in GSH concentration with Cd treatment was observed. A significantly decreased GSH/GSSG ratio was observed only in the 200-μM Cd treatment. These results suggested that a high GSH/GSSG ratio could be maintained by the addition of NAC in the plants exposed to Cd treatment.

When plants are exposed to toxic metal stress, intracellular metal-binding proteins and peptides can be synthesized to defend against possible damage [54]. Cd as a class B metal preferentially binds to S-containing ligands. Accordingly, apart from the cysteine-containing peptides (GSH and PCs), cysteine-rich proteins could be effective chelators for Cd ions. Our results demonstrated that the Cd treatment not only increased levels of non-protein thiols, but also increased the levels of protein thiols in *S. nigrum* roots (Table 4). Furthermore, by using size-exclusion chromatography, we found that NAC treatment could enhance Cd-binding protein synthesis (Fig. 4). In the plants exposed to 50 μM Cd, the addition of 500 μM NAC increased the height of the UV-absorbing peak, and Cd concentrations in the 55–75 ml volumes increased in the roots of *S. nigrum*. The peak between 55 and 75 ml may represent high-molecular-weight proteins, and the second peak (145–185 ml) a low-molecular-weight substance. The results suggest an involvement of NAC in the Cd tolerance mechanism through increasing the biosynthesis of Cd-binding proteins. Further detailed work is needed to explore the exact components of Cd-binding complexes and their roles in Cd tolerance.

In conclusion, our results suggest that Cd treatment can induce lipid peroxidation and increase the accumulation of H_2O_2 and $O_2^{\bullet-}$ in the roots of *S. nigrum*. It is suggested that the majority of Cd-induced H_2O_2 production in the roots of *S. nigrum* may be via PM NADPH oxidase. In addition to NADPH oxidase, POD also may contribute to the production of H_2O_2. Exogenous NAC treatment could protect plants from Cd-induced oxidative stress damage and increase Cd tolerance in *S. nigrum*. This protection seems to be achieved via increased GSH biosynthesis rather than via increased CAT and APX activities.

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