

Alleviation of cadmium toxicity to Cole (*Brassica campestris* L. Cruciferae) by exogenous glutathione

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Abstract. In this study, we determined the influence of exogenous GSH on cadmium toxicity to cole. GSH addition had beneficial effect on plant development and growth, especially on aboveground biomass and root length. Despite that exogenous GSH insignificantly promoted Cd uptake by the plant, it could decrease of Cd root-to-shoot transport and ameliorate Cd toxicity to the plant. At 6 mg Cd kg⁻¹ soil, GSH addition well countered the Cd-induced significant reduction in CAT activity, but only insignificantly decreased MDA content, suggesting exogenous GSH might indirectly protect plant against oxidative stress via regulating antioxidative enzyme activities. However, at 12 mg Cd kg⁻¹ soil, GSH application insignificantly increased the antioxidant activities but significantly decreased MDA content, indicating external GSH could directly participate in removing radical oxygen species. The results suggest exogenous GSH may have the potential of decreasing Cd accumulation in the edible parts of cultivars and alleviating Cd toxicity.

1. Introduction

Inorganic pollution is the main type of soil contamination in China, accounting for over 82% of the sample points exceeding standard value, and the percent of cadmium (Cd) content exceeding standard value ranks first with 7.0%. Cd contamination in soils is becoming a serious problem due to its absorption by plants and transference to human food chain [1]. Cd is a non-essential and toxic metal ion for organisms, and it can induce Cd toxicity in plants such as growth inhibition, chlorosis, and alterations of morphological, physiological, or biochemical properties [2,3]. Studies on different plant species have revealed that the exposure to toxic levels of Cd triggers membrane injury and alteration of antioxidative defense system due to oxidative stress caused by reactive oxygen species (ROS) [4,5]. Correspondingly, plants also have evolved intrinsic mechanisms to regulate ROS levels [6,7]. These mechanisms include enzymatic and non-enzymatic defense systems. As a part of defensive mechanism, antioxidative enzymes, especially superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6) play an important role in scavenging ROS. SOD is an essential component of antioxidation system in plants as it can dismutate O₂^{•−} to H₂O₂ and O₂. CAT is one of the key enzymes for the detoxification of H₂O₂ via two electron transfer [1]. Therefore, it is imperative to determine the changes in antioxidative enzymes in order to verify the hypothesis that these enzymes can mitigate Cd stress in plants.



Reduced glutathione (γ -Glu-Cys-Gly, GSH), a non-enzymatic antioxidant, is a major low-molecular-weight thiol tripeptide implicated in a variety of life processes and involves in defense system against environmental stresses including heavy metal (HM) [8,9]. GSH can act indirectly as a substrate for some enzymes or phytochelatins (PCs) to alleviate HM toxicity. It is a substrate for glutathione peroxidase (GPX) and glutathione transferase (GST), which are involved in the removal of ROS [10]. GSH is also a precursor for the synthesis of PCs, which are a set of novel heavy metal-binding peptides [11]. This has documented that an increase in intracellular GSH concentration enhanced Cd tolerance and increased Cd accumulation in *Brassica campestris* [12]. Although GSH indirectly protects plants against HM toxicity by functioning as a substrate, it can also act directly as an antioxidant to detoxify ROS (especially H_2O_2) associated with biotic and abiotic stress through the ascorbate-glutathione cycle (AsA-GSH cycle) [9]. It plays an important role in maintaining cellular homeostasis as a redox couple since it allows fine-tuning of cellular redox environment under normal conditions and upon the onset of stress. GSH accumulates in response to increased ROS, or to compensate for decrease in the defense capability of other antioxidants and GSH levels are constitutively higher in plants adapted to stress conditions [13,14]. Studies about the effect of intracellular GSH on heavy metal metabolism and toxicity have indicated that GSH can serve to detoxify peroxides, maintain the correct three-dimensional structure of many proteins, synthesize phytochelatins (PCs), mediate enzyme activities, etc [15,16].

Previous studies have revealed that exogenous GSH can trigger a positive effect on antioxidant defense system against abiotic stress. External GSH conferred a high temperature stress tolerance in mung bean (*Vigna radiate* L.) by increasing chlorophyll content and enzyme activity (e.g. CAT, SOD, GPX) and decreasing MDA and H_2O_2 content, resulting in better physiological performance [17]. Exogenous application of GSH lessened the adverse effects of Pb on photosynthetic activities and improved cotton's tolerance via reducing lipid peroxidation and antioxidant enzyme activities [18]. GSH application could alleviate the reduction of aboveground biomass, enhance Pb accumulation in shoots and roots, and induce the synthesis of phytochelatins (PCs) [19]. Exogenous GSH appeared to limit the toxicity of herbicide and seemed to overcome the stress status due to enhancing contents of photosynthetic pigments and activities of antioxidant enzymes [20]. However, the mechanism of toxicity alleviation by exogenous GSH in plant under various levels of Cd stress has not been well documented.

Thus, in this study, we chose to investigate the mechanism of toxicity alleviation by exogenous GSH in cole (*Brassica campestris* L. Cruciferae) exposed to various levels of Cd stress. Cole is a most frequently seen vegetable in the dishes during the hot summer days in China for its low energy but high fiber. We hypothesized that (1) GSH addition will alleviate Cd toxicity, and would be reflected in the negative contribution of malondialdehyde (MDA) and in the positive contribution of plant growth; (2) Extracellular GSH can counteract Cd-induced alterations of certain antioxidant enzyme activities.

2. Methods and materials

Table 1. Pot experimental design in greenhouse

Treatment		Content ($\text{mg}\cdot\text{kg}^{-1}$ soil)				
		Cd	GSH	N	P	K
Control	CK	0	0	150	200	300
Group1	Cd6	6	0	150	200	300
	Cd6+GSH	6	30			
Group2	Cd12	12	0	150	200	300
	Cd12+GSH	12	30			

The experiment was conducted in Shenyang Station of Experimental Ecology, Chinese Academy of Sciences ($41^{\circ}31'N$ and $123^{\circ}41'E$), which is located in the North Temperate Zone with a semimoi

continental climate. The tested soil is grey meadow soil, and its soil bulk density, pH, total organic carbon (TOC), total N and total P were 1.33 g·cm⁻³, 7.63, 1.67%, 0.95g·kg⁻¹ and 0.45 g·kg⁻¹, respectively. These soils were passed through a 4-mm sieve. 5.5 kilograms of soils were mixed with CdCl₂·5H₂O or CdCl₂·5H₂O accompanied by GSH as listed in Table 1, filled into plastic pots, applied with soluble fertilizers and equilibrated for 2 weeks. Three uniform seedlings of cole (*Brassica campestris* L. Cruciferae) were transplanted into each pot. Each treatment was replicated five times. No fertilizers were added throughout the cultivation.

After 60 days, the cole was harvested and the experiment was terminated. After being dried with filter papers, the plant samples were dried at 105°C for 10 min, then at 80°C until constant weight, and weighed for dry weight (DW). Dry plant samples were grounded to powders, and digested with a concentration acid mixture of HNO₃/HClO₄ (3:1, v/v) [21]. The Cd concentration in plant tissues was determined using the AAS method [22].

Lipid peroxidation was estimated by the concentration of malondialdehyde (MDA). MDA content was exactly and estimated with minor modification of the method of Ortega-Villssante et al. (Tieheng et al, 2005). The activities of superoxide dismutase (SOD) and catalase (CAT) were measured as described by Chance and Maehly [23].

The translocation factor (TF) indicated the ability of plants to translocate heavy metals from roots to the leaves [1]. It was calculated as:

$$TF = \frac{\text{the metal concentration in leaves}}{\text{the metal concentration in roots}} \quad \text{Eq.}$$

All data obtained from the experiments were performed with SPSS 18.0 and Excel 2007.

Table 2. Statistics for plant height, root length, aboveground biomass and underground biomass of tested plants, each with five replicates. Value in tables are presented by mean±SE. Data were compared with single factor analysis of variance. Different lowercase letters in a row mean significant differences (P<0.05).

Treatment		Plant height [cm·plant ⁻¹]	Root length [cm·plant ⁻¹]	Aboveground iomass [g·plant ⁻¹]	Underground biomass [g·plant ⁻¹]
Control	CK	23.60±2.47 a	14.20±2.14 a	16.12±1.36 a	7.68±0.45 a
Group1	Cd6	19.20±1.80 c	13.27±2.14 b	13.52±0.80 b	7.28±0.66 b
	Cd6+GSH	21.73±1.73 b	14.73±1.83 a	15.98±1.27 a	7.16±0.26 b
Group2	Cd12	20.33±0.70 c	13.10±1.71 c	11.86±1.20 c	6.97±0.29 b
	Cd12+GSH	20.93±1.24 bc	14.30±2.23 a	15.56±1.76 a	7.22±0.24 b

3. Results

As shown in Table 2, GSH application could improve development and growth of cole under Cd stress. For plant height, the supply of GSH led to a significant increase at 6 mg Cd kg⁻¹ soil, and Cd also exerted a profoundly deleterious effect on all the treatments. Furthermore, the application of GSH significantly improved root length and increased aboveground biomass in comparison with the treatments with no GSH supply, and Cd did not have an obvious effect on the treatments with GSH addition. For underground biomass, the application of GSH resulted in a slight increase (without Group 1) in the same Cd treatments, and the biomass of all the treatments was evidently lower than that of the control.

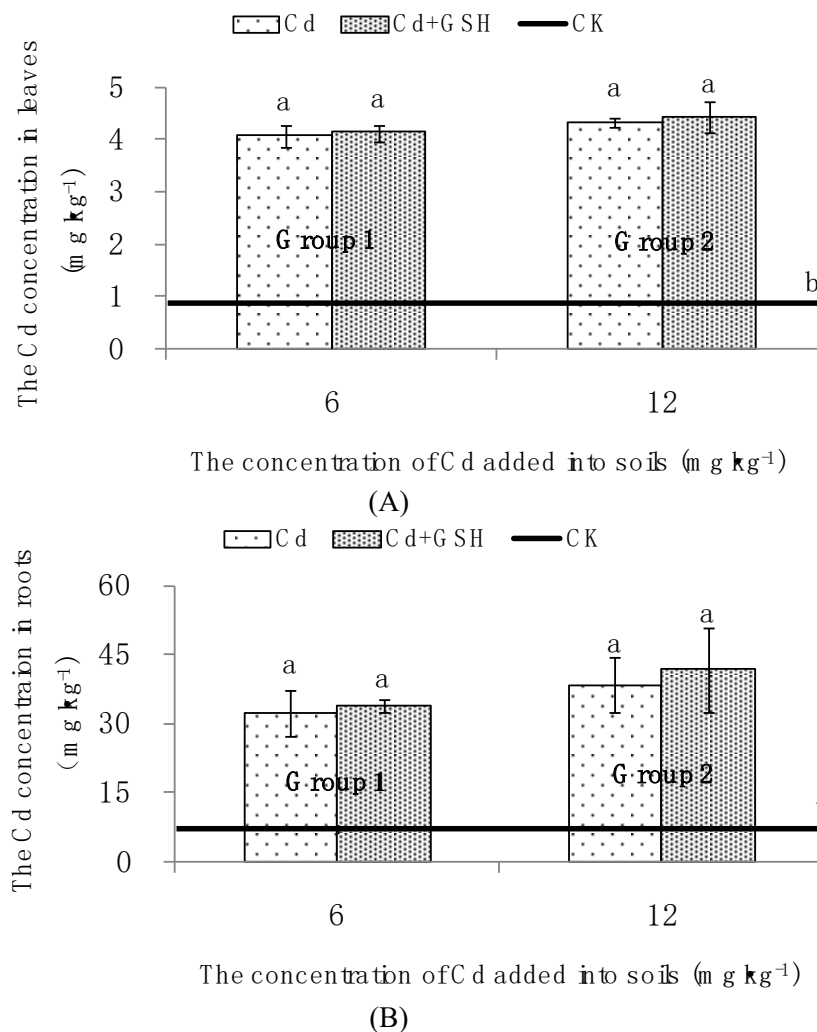


Figure 1. Effects of GSH application on Cadmium contents in leaves (A) and roots (B) of cole in soil contaminated with different concentrations of Cd. Error bars represent SE for $n=5$. Horizontal line represents the control (CK). The different letters indicate a significant difference at $P<0.05$.

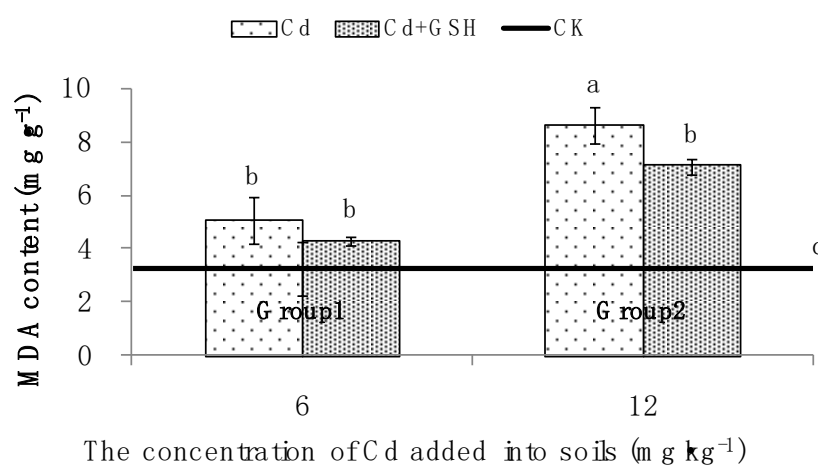


Figure 2. Effect of GSH application on malondialdehyde (MDA) content of cole in soil contaminated with different concentrations of Cd. Error bars represent SE for $n=5$. Horizontal line represents the control (CK). The different letters indicate a significant difference at $P<0.05$.

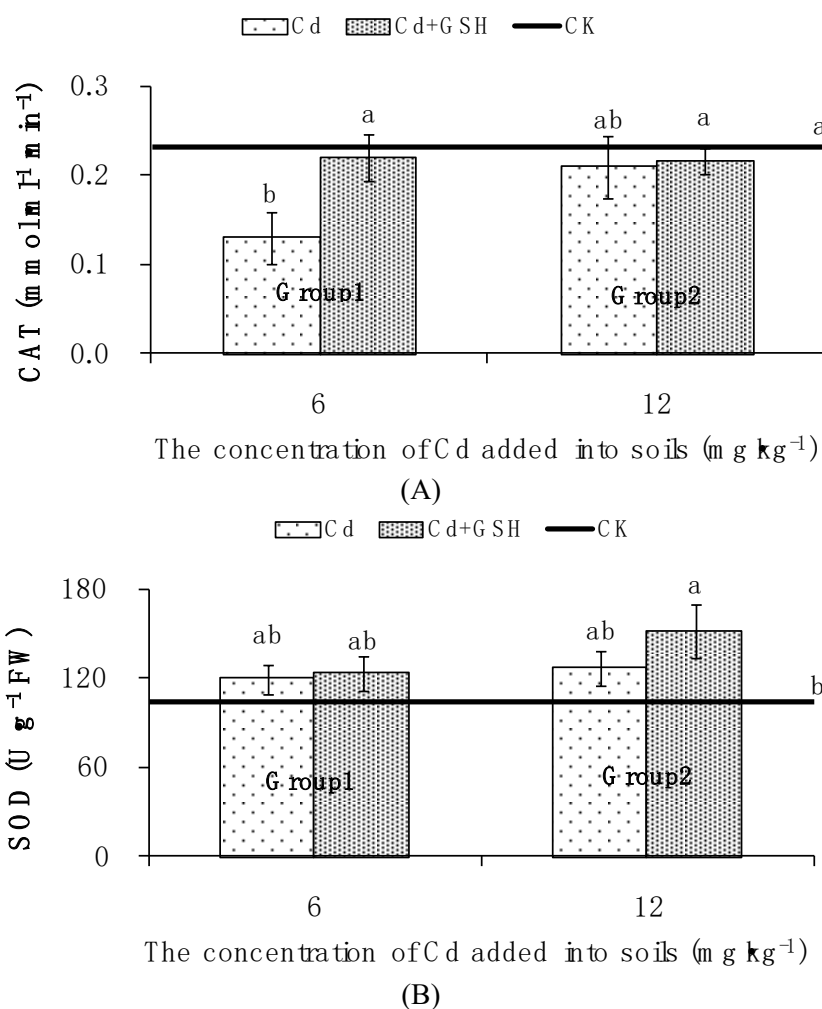


Figure 3. Effect of GSH application on the activities of superoxide dismutase (SOD) (A) and catalase (CAT) (B) of cole in soil contaminated with different concentrations of Cd. Error bars represent SE for $n=5$. Horizontal line represents the control (CK). The different letters indicate a significant difference at $P<0.05$.

The Cd concentrations in leaves and roots increased with increasing Cd exposure (Figure 1). GSH application promoted Cd accumulation, but the plants exposed to GSH and Cd did not exhibit a significantly higher Cd content than those exposed to Cd only (except for the control). Cd contents of both leaves and roots in all the treatments were obviously higher than those of the control, and increased progressively with the increasing addition of Cd in soil. Cd content in leaves was significantly lower than that of roots in the same treatment. A large increase in Cd contents occurred in leaves and roots at 12 mg Cd kg^{-1} soil, and similarly after GSH application.

The level of lipid peroxidation in cole leaves was measured in terms of MDA content (Figure 2). MDA contents in the both experimental groups were significantly higher as compared to the control, and increased with the increasing Cd concentration. Under Cd exposure, MDA content increased significantly from 6 mg Cd kg^{-1} soil to 12 mg Cd kg^{-1} soil. Under GSH and Cd exposure, MDA content at 6 mg Cd kg^{-1} soil was significantly lower than that at 12 mg Cd kg^{-1} soil. GSH application induced detoxification of Cd as evidenced by the decrease in MDA content, although the decrease was only significant when Cd was applied at 12 mg Cd kg^{-1} soil.

As shown in Figure 3. A, the enzyme activities of superoxide dismutase (SOD) in the both groups were higher than that in the control. Particularly, SOD activity peaked at 12 mg Cd kg^{-1} soil with GSH

addition and was significantly higher than that in the control. In the both groups, SOD activities under stress of different levels of Cd with GSH addition were not significantly higher than those without GSH addition.

Catalase (CAT) activities in the control were higher than those in the both groups, and significantly higher than that at 6 mg Cd kg⁻¹ soil without GSH application (Figure 3. B). Under Cd stress without GSH application, CAT activities increased with increasing the level of Cd exposed, but showed no significant difference. Under Cd stress with GSH application, CAT activities did not show any significant difference. At 6 mg Cd kg⁻¹ soil, CAT activity exposed to Cd and GSH exhibited a significant increase than that exposed to Cd.

4. Discussion

Reduced glutathione (γ -Glu-Cys-Gly, GSH), a major low-molecular-weight thiol tripeptide, is a nonenzymatic antioxidant. The thiol group of GSH is important in formation of mercaptide bond with metal and for reacting with selected electrophiles [11]. This chemical reactivity makes GSH particularly suitable to serve a broad range of biochemical functions in all organisms. Furthermore, GSH also plays an important role in detoxification processes due to control of H₂O₂ levels through the AsA-GSH cycle, and functions directly as a free radical scavenger by reacting with $1O_2$, $O_2^{\bullet-}$ and $\bullet OH$ [13]. Because GSH has been documented in enhancing plant tolerance to heavy metals [24], we applied it in all experimental groups as a modifier to alleviate Cd toxicity.

Table 3. Statistics for Cd accumulation of tested plants in contaminated soils treated with Cd or Cd and GSH.

	Treatment	TF
Control	CK	0.124
Group1	Cd6	0.131
	Cd6+GSH	0.122
Group2	Cd12	0.113
	Cd12+GSH	0.106

Being a non-essential and toxic metal ion for organisms, Cd can exhibit detrimental effects on plant growth and yield [25]. In the present study, the statistics for plant height, root length, aboveground biomass and underground biomass were clearly impaired under stress from 6 mg Cd kg⁻¹ soil to 12 mg Cd kg⁻¹ soil in comparison with the control, and GSH addition could mitigate the negative effects of Cd on plant growth and development (Table 2). Several other studies have also documented that Cd can induce visible symptoms of phytotoxicity, such as plant growth inhibition and yield reduction [17,26]. Furthermore, Cd is a major heavy metal threatening food safety due to its high soil-plant mobility and easy accumulation in plant tissues (especially edible parts). Single Cd-treatments significantly increased Cd uptake in roots and leaves compared to the control, GSH addition could slightly enhance Cd accumulation, and the promoting effect of GSH on Cd accumulation in roots was larger than that in leaves (Figure. 1). Translocation factor (TF) is used to demonstrate the ability of Cd translocation from root to shoot. The lower TF with GSH application (Table 3) suggested that GSH could weaken the ability to translocate Cd to leaves. Similarly, several reports have suggested that GSH application can lessen the adverse effects of heavy metals and improve plant's tolerance. Fanrong suggested that exogenous application of GSH in the culture solution obviously alleviated the reduction of plant growth, and caused significant decrease of Cr root-to-shoot transport in the Cr-stressed rice plants [27]. Shin-Ichii N. found that GSH application significantly inhibited root-to-shoot Cd translocation via xylem vessels in oilseed rape. Based on lower TF and higher growth indicators (e.g. plant height, root length and aboveground biomass), GSH has the potential of decreasing Cd accumulation in the edible parts of cultivars to reduce risks associated with Cd toxicity and maintain sustainable crop production [28].

The role of external GSH addition in alleviation of Cd-induced damage was also revealed by its effects on MDA and some antioxidative enzymes. Cd may initiate ROS generation and the process of lipid peroxidation, resulting accumulation of MDA [29], which is a product of cell membrane lipid peroxidation. The change in MDA content indicates the degree of antioxidant damage under abiotic stress. The present results revealed that Cd caused a dramatic increase in MDA content in cole leaves (Figure 2), indicating an occurrence of high oxidative stress due to ROS generation. However, GSH addition reduced MDA accumulation, suggesting that GSH may enhance antioxidant capacity in Cd-stressed plants. Furthermore, GSH addition increased the activities of CAT and SOD. CAT is one of the key enzymes for the detoxification of H₂O₂ via two electron transfer. SOD is an essential component of antioxidation system in plants as it can dismutate O₂•⁻ to H₂O₂ and O₂ (Zhou et al., 2009). In our study, the significant reduction in CAT activity induced by Cd stress (6 mg Cd kg⁻¹ soil) was well countered by the addition of GSH. The results are agreed with Fei C.[30]. Thus, we conclude that in our experiment exogenous GSH might indirectly protect plant against oxidative stress resulted from Cd toxicity via regulating antioxidative enzyme activities.

Another antioxidative defense strategy of GSH is to react with ROS as an antioxidant as evidenced in our study. Under the high Cd stress (12 mg Cd kg⁻¹ soil), the promoting effect of GSH on activity of SOD was larger than on CAT (Figure. 3), indicating that SOD is over expressed in dismutation of O₂•⁻ to H₂O₂, resulting in lipid peroxidation due to H₂O₂ accumulation. More importantly, the application of GSH, which functioned as an intracellular ROS-scavenger through the AsA-GSH cycle [13], resulted in a significant reduction in MDA content under high Cd stress. Our results agree with several previous findings [31]. Thus we speculate that external GSH could directly participate in scavenging ROS as an antioxidant.

5. Conclusions

In this study, the influence of exogenous GSH on Cd-induced oxidative stress was investigated. GSH addition could promote development and growth of plants, and ameliorated lipid peroxidation. Exogenous GSH successfully regulated the activities of CAT and SOD, and decreased MDA contents. We speculated that exogenous GSH not only acts indirectly in regulating ROS-scavenging enzyme activity but also acts directly as an antioxidant to remove ROS. The application of additional GSH may provide an effective alternative to alleviate oxidative stress resulted from Cd toxicity.

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