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# Alleviating Effects of Exogenous Glutathione, Glycinebetaine, Brassinosteroids and Salicylic Acid on Cadmium Toxicity in Rice Seedlings (*Oryza Sativa*)

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## Abstract

A hydroponic experiment was conducted to study the ameliorative effects of 24 h pretreatment with exogenous glutathione (GSH), glycinebetaine (GB), brassinosteroids (BRs) and salicylic acid (SA) upon 50 μM cadmium (Cd) stress to rice seedlings. The results showed that Cd caused a significant reduction in seedling height, chlorophyll content and biomass, the activity of POD in stems, and shoot Mn, shoot/root Zn and root Cu concentration, but improved SOD and POD activities in leaves with elevated MDA accumulation and Fe concentration. Pretreatment with 100 μM GSH, GB or SA greatly alleviated Cd-induced growth inhibition and suppressed Cd-induced MDA accumulation. Compared with Cd alone treatment, pretreatment of GSH, GB and SA markedly increased chlorophyll content; reduced shoot Cd concentration, and GSH also decrease root Cd content. GSH, GB and SA pretreatments counteracted the pattern of alterations in certain antioxidant enzymes induced by Cd, e.g. significantly suppressed Cd-induced increase of leaf SOD activity, GB and BRs also significantly decreased leaf POD activity; GSH significantly elevated the depressed stem POD and SOD activities; and GB elevated stem SOD activity. Compared with Cd alone, GSH pretreatment significantly relieved Cd-induced reduction in Cu or increase in Fe concentration in leaves; GB pretreatment decreased Cd-induced Fe enhancement; SA pretreatment markedly increased shoot Fe, but reduced Mn concentration. Although BRs pretreatment increased plant dry biomass with no effect on chlorophyll content and MDA accumulation, significantly increased Cd concentration both in shoots and roots.

**Keywords:** Alleviation; Brassinosteroids (BRs); Cadmium (Cd); Glutathione (GSH); Glycinebetaine (GB); Rice (*Oryza sativa* L); Salicylic acid (SA)

## Introduction

Heavy metal contamination in soil could resulted in inhibition of plant growth and yield reduction, and even pose a great threat to human health via food chain [1]. Among heavy metals, Cadmium (Cd) in particular causes increasingly international concern [2]. Cd-contaminated soil results in considerable accumulation of Cd in edible parts of crops, and then it enters the food chain through the translocation and accumulation by plants [3-6]. Cereals, especially rice, the staple food in East Asia, is a major source of heavy metal intake [7]. For example, rice, a staple crop for Japanese, was estimated to represent 36-50% of the total oral intake of Cd for Japanese population during 1998-2001 [8]. Correspondingly, it is imperative to find reliable approaches to decrease Cd accumulation in rice aimed for decreasing Cd content in human food.

Previous studies have shown that Cd toxicity causes oxidative stress as accumulation of reactive oxygen species (ROS) and subsequent production of membrane lipid peroxides [9,10]. Reduced glutathione (GSH, γ-Glu-Cys-Gly), an essential metabolite and regulator in plants, plays important role in the cellular defense against abiotic stress [11]. It was reported that GSH can significantly alleviate the Cd induced growth inhibition [12,13]. Zhu et al. [14,15] observed that the over-expression of glutathione synthetase and γ-glutamine homocysteine synthase can markedly improve the tolerance to Cd stress in indian mustard. Salicylic acid (SA) is a cellular signal element and plays an important role against biotic and abiotic stress in plants [16]. Barley seeds were presoaked in SA for 6 h can significantly reduce the Cd content and alleviate the lipid peroxidation in the seedling under Cd stress [17]. Moreover, SA can reduce the damage of photosynthesis system caused by Cd in maize [18]. Glycinebetaine (GB), commonly found in higher plants, is a very important osmoregulation substance. Under abiotic stress, such as drought and salinity, its concentration would increase [19]. And the foliar spray of GB could markedly alleviate the water stress [20]. Furthermore, the exogenous GB could also improve the tobacco tolerance against Cd stress [21]. Brassino steroids (BRs) are a new class plant steroidal hormone. It plays an important role in a widely spectrum of physiological processes, such as leaf epinasty, pollen tube growth and stem elongation [22]. In addition, BRs has important function against environmental stress. Now, BRs attracted more attention in adaptive response to abiotic stress, particularly in respect to chilled stress [23], water stress [24], and heat stress [22]. Meanwhile, BRs application can decrease the Cd-induced stress through enhancing antioxidant systems in *Brassica juncea* [25]. On the whole, application of GSH, GB, SA and BRs have been widely investigated in alleviating abiotic stresses, including Cd toxicity. However, effects of pretreatment of GSH, GB, SA and BRs against Cd toxicity have not been reported except SA, which could alleviate Cd-induced oxidative damage in rice root. In this study, a hydroponic experiment was conducted to determine: whether GSH, GB, BRs and SA could alleviate Cd toxicity, whether GSH, GB, BRs and SA application altered uptake and translocation of Cd and other elements in rice plants, and whether antioxidant enzymes were involved in the mitigation-measure-mediated protective responses of rice plants exposed to Cd stress.

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## Materials and Methods

### Culture condition and treatments

A hydroponic experiment was carried out in the greenhouse of Huajiachi campus, Zhejiang University, Hangzhou, China. Rice Xiushui63 (*Oryza sativa* L. *Japonica* unwaxy) seeds were surface sterilized with 2% $H_2O_2$ for 20 min, and fully rinsed with deionized water, soaked in deionized water at room temperature for 2 days, then germinated for 1 day at 35°C. Germinated seeds were sown in sterilized sand bed and kept in an incubator at 30°C-day/26°C-night under 85% relative humidity for 10 days. At two-leaf stage, the uniform healthy plants were transplanted into plastic containers filled with 5-L basic nutrient solution. The container was covered by a polystyrol plate with 7 evenly spaced holes (2 plants per hole) and placed in greenhouse. After 7 days of transplanting, rice seedlings were pre-treated with 100 μM GSH, 100 μM GB, 10 μM BRs or 100 μM SA for 24 h and then exposed to 50 μM Cd in nutrient solution for 5 d. There were six treatments: (1) Control (basic nutrient solution), (2) Cd (50 μM Cd), and (3) pre-GSH+Cd, (4) pre-GB+Cd, (5) pre-BRs+Cd, (6) pre-SA+Cd, which were pretreated with 100 μM GSH, 100 μM GB, 10 μM BRs, 100 μM SA for 24 h, respectively, then exposed to 50 μM Cd for 5 d. The composition of the basic nutrient solution was (mg $l^{-1}$): $NH_4NO_3$ 57.1; $NaH_2PO_4 \cdot 2H_2O$ 25.2; $K_2SO_4$ 44.7; $CaCl_2$ 55.4; $MgSO_4 \cdot 7H_2O$ 202.5; $MnCl_2 \cdot 4H_2O$ 0.94; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.05; $H_3BO_3$ 0.59; $ZnSO_4 \cdot 7H_2O$ 0.03; $CuSO_4 \cdot 5H_2O$ 0.02; Fe-citrate 7.44. The experiment was laid in a completely randomized design with 4 replicates. The nutrient solution pH was adjusted to 5.6 ± 0.1 with NaOH or HCl as required. Plant samples were collected after 5 d Cd exposure. The fresh roots and the upper second fully expanded leaves were sampled, and immediately frozen in liquid nitrogen and stored frozen at -80°C for further analyses or directly used for the determination of anti oxidative enzyme activities and MDA content.

### Measurements of chlorophyll content and plant growth parameters

The upper second fully leaves were selected to measure SPAD (Soil Plant Analysis Development) value with three replicates. And chlorophyll content of the second uppermost fully expanded leaves was determined according to Cai et al. [12]. After measuring plant height and root length, roots were soaked in 20 mM $Na_2$-EDTA for 3 h and rinsed thoroughly with deionized water. Plants were separated into roots and tops (shoots and leaves), and then dried at 80°C and weighed. Dried shoots and roots were powdered and weighted, then ashed at 550°C for 12 h. The ash was digested with 5 ml 30% $HNO_3$, and then diluted with deionized water. Metal concentrations were determined using a flame atomic absorption spectrometry (SHIMADZU AA-6300, Japan) [26].

### Assay of enzyme activities and MDA content

For the determination of enzyme activities, plant tissue was homogenized in 6 ml 50 mM sodium phosphate buffer (PBS, pH 7.8,) using a pre chilled mortar and pestle, then centrifuged at 10,000×g for 20 min at 4°C. The supernatant was used for enzyme assay. Superoxide dismutase (SOD), peroxidase (POD) and MDA content were measured according to Zeng et al. [27].

### Statistic analysis

Statistical assay were performed with SPSS version 17.0. One-way ANOVA was carried out by the Duncan's Multiple Range Test (SSR) to analysis the difference of significance among treatments.

## Results

### Effect of GSH, GB, BRs and SA pretreatment on growth of Cd-stressed rice

As shown in table 1, exposure to Cd caused obvious inhibition of growth traits. To evaluate the alleviating effects of glutathione (GSH), glycinebetaine (GB), brassino steroids (BRs), salicylic acid (SA) pretreatment for 24 h, we adopt the following formula-based integrated score: SPAD×0.5+shoot height×0.125+root length×0.125+fresh weight×0.125+dry weight×0.125 (Table 1). There is a positive correlation between alleviating effect of different treatments and the integrated scores. According to the integrated scores, the alleviation effect is in order of GSH>GB>SA>BRs. GSH, GB, SA greatly alleviated the Cd-induced growth inhibition. Compared with Cd treatment, GSH pre-treatment increased SPAD value, root length, shoot dry weight and root dry weight by 57.2%, 26.1%, 15.5% and 9.8%, respectively; GB pre-treatment increased by 41.0%, 19.6%, 30.8% and 27.4%, respectively; SA pre-treatment increased by 42.2%, 25.0%, 38.5% and 31.4%, respectively.

### Effect of GSH, GB, BRs and SA pretreatment on chlorophyll content

Exposure of the rice seedlings to 50 μM Cd for 5 d markedly reduced Chl and b contents by 11.8% and 14.0%, respectively, compared with control. GSH, GB and SA pretreatment for 24 h greatly relieved Cd-induced reduction in Chl a and Chl b content and the values returned close to or even higher than control, c.f. increased by 43.1%, 19.5% and 24.5% (Chl a), and 48.7%, 22.4% and 26.7% (Chl b), respectively,

| | SPAD value | | Shoot height (cm) | | Root length (cm) | | Fresh weight (g per plant) | | | | Dry weight (g per plant) | | | | Interqrated Score | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Shoot | | Root | | Shoot | | Root | | | |
| Control | 20.4 | c | 17.7 | a | 12.0 | a | 121 | a | 66 | a | 16 | ab | 5.7 | bc | 40.0 | a |
| *pre*-GSH+Cd | 26.1 | a | 16.3 | ab | 11.6 | a | 102 | b | 63 | a | 15 | b | 5.6 | c | 39.7 | a |
| *pre*-GB+Cd | 23.4 | b | 16.3 | ab | 11.0 | a | 113 | ab | 66 | a | 17 | ab | 6.5 | ab | 40.4 | a |
| *pre*-BRs+Cd | 16.1 | d | 16.5 | ab | 11.8 | a | 109 | ab | 68 | a | 17 | ab | 6.6 | a | 36.7 | b |
| *pre*-SA+Cd | 23.6 | b | 16.1 | b | 11.5 | a | 115 | ab | 66 | a | 18 | a | 6.7 | a | 41.0 | a |
| Cd | 16.6 | d | 15.4 | b | 9.2 | b | 98 | b | 60 | ab | 13 | c | 5.1 | d | 33.4 | c |

**Table 1:** Effect of GSH, GB, BRs and SA on SPAD value, and growth traits of rice seedlings exposed to Cd stress.
Rice seedlings were pre-treated with 100 μM GSH, 100 μM GB, 10 μM BRs and 100 μM SA for 24 h and then exposed to 50 μM Cd in nutrient solution for 5 d. Control and Cd correspond to basic nutrient solution and 50 μM Cd; *pre*-GSH+Cd, *pre*-GB+Cd, *pre*-BRs+Cd and *pre*-SA+Cd correspond to 5 d 50 μM Cd exposure after 24 h pretreated with 100 μM GSH, 100 μM GB, 10 μM BRs and 100 μM SA, respectively. The same as below. Different letters indicates significant differences among treatments (P<0.05). Error bars represent SD values.
Integrated score=absolute values of [SPAD value×0.5+shoot height×0.125+root length×0.125+fresh weight×0.125+dry weight×0.125].

compared with Cd alone treatment (Figure 1). No effect was detected in BRs pretreatment on Chla and Chl b content.

## Effect of GSH, GB, BRs and SA pretreatment on SOD and POD activity in leaf, stem and root of rice

Cd stress caused 54.7% increase in leaf SOD activity, while no effect on stem and root SOD activity relative to control (Figure 2). Pretreated with GSH, GB, BRs and SA significantly lowered Cd-mediated increase in SOD activity by 17.9%, 22.5%, 15.6% and 24.0% in leaves, but no effect on stems and roots except stems of GSH and GB, compared with Cd alone treatment. Concerning POD activity, Cd stress caused 38.9% increase in leaves, but 37.1% reduction in stems and no effect on roots (Figure 2). In leaves, GB and BRs pretreatment significantly decreased POD activity and dropped it back to control level, c.f. decreased by

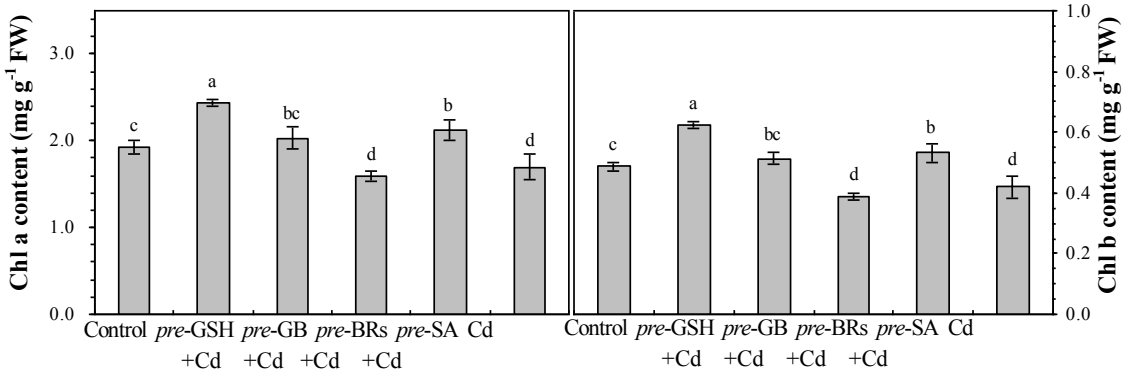


**Figure 1:** Effect of Cd on chlorophyll content and as affected by pretreatment of GSH, GB, BRs and SA in rice seedlings. Rice seedlings were pre-treated with 100 μM GSH, 100 μM GB, 10 μM BRs and 100 μM SA for 24 h and then exposed to 50 μM Cd in nutrient solution for 5 d. Control and Cd correspond to basic nutrient solution and 50 μM Cd; *pre*-GSH+Cd, *pre*-GB+Cd, *pre*-BRs+Cd and *pre*-SA+Cd correspond to 5 d 50 μM Cd exposure after 24 h pretreated with 100 μM GSH, 100 μM GB, 10 μM BRs and 100 μM SA, respectively. Error bars refer to SD value. Different letters indicate significant differences (P<0.05) among treatments.

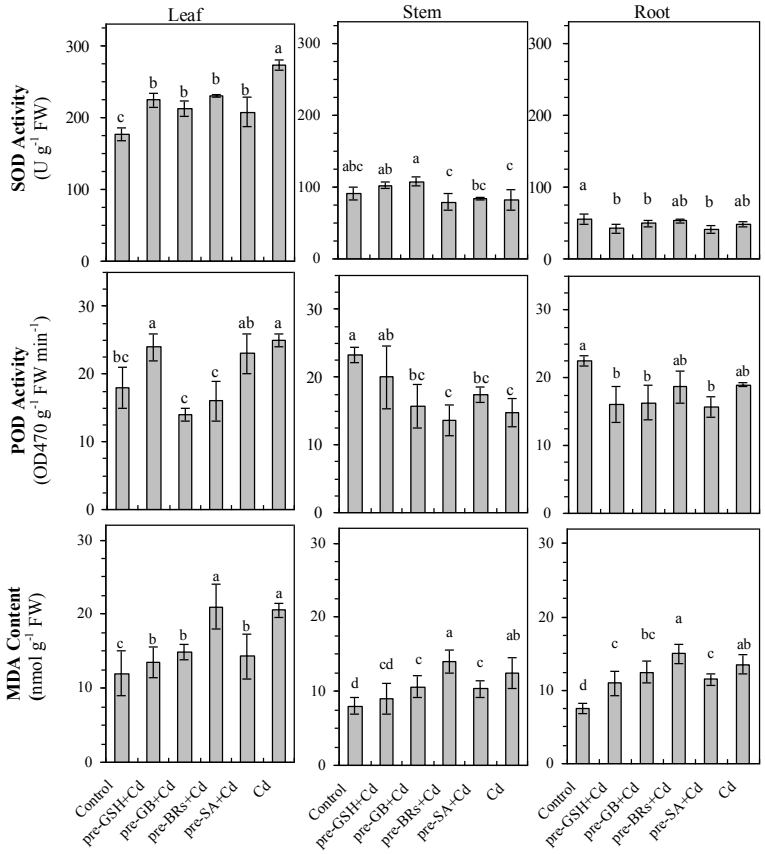


**Figure 2:** Effect of GSH, GB, BRs and SA on SOD, POD activities and MDA content in leaf, stem and root of rice seedlings exposed to Cd stress. Different letters indicate significant differences (P<0.05) among the 6 treatments.

44.0% and 36.0%, respectively, compared with Cd alone treatment. In stems, only GSH pretreatment markedly elevated Cd-mediated decrease POD activity, increased by 36.4%. Yet, no effect was observed in other 3 mitigation treatments. In roots, GSH, GB, BRs and SA pretreatment kept POD activity similar with Cd alone treatment.

### Effect of GSH, GB, BRs and SA pretreatment on MDA content in leaf, stem and root of rice

In comparison with control, Cd alone treatment induced 70.8%, 56.2% and 80.0% more MDA accumulation in leaves, stems and roots, respectively, over the control (Figure 2). GSH and SA pretreatment significantly decreased MDA content by 34.1% and 30.2% in leaves, 28.0% and 17.6% in stems, and 18.5% and 14.8% in roots, respectively, compared with Cd alone treatment. GB pretreatment inhibited MDA accumulation in leaves and stems, but no effect on root MDA content. However, BRs pretreatment did not mitigate MDA overproduction induced by Cd stress.

### Effect of GSH, GB, BRs and SA pretreatment on mineral concentration

As shown in tables 2 and 3, exposure to Cd alone caused obvious increase in Fe in shoots/roots, and reduction in shoots/roots Zn, shoots Mn and root Cu, compared with control. GSH, GB and SA pretreatment significantly reduced shoot Cd concentration by 46.1%, 22.0% and 20.5%, respectively, but no effect on root except GSH pretreatment which markedly decreased Cd concentration by 24.3%. On the contrary, BRs pretreatment significantly increase the shoot and root Cd concentration. In leaves, GSH pretreatment significantly decreased Fe and Mn concentration by 23.1% and 13.2%, but increase Cu by 26.9%, compared with Cd alone. GB pretreatment decreased Fe and Mn by 25.6% and 23.4%, respectively. BRs pretreatment markedly elevated Cu by 26.7%, but decreased Fe and Zn by 28.2% and 15.8%, respectively. Moreover, SA pretreatment significantly increased Fe by 30.8%, but decreased Mn by 14.6%. In roots, GSH pretreatment decreased Mn and Cu by 30.3% and 12.5%, compared with Cd alone treatment. GB decreased Fe and Mn by 22.8% and 33.9%, respectively. BRs significantly decreased Fe, Mn and Cu by 40.0%, 34.9% and 18.2%, respectively. SA only decreased Mn by 20.2%.

| Treatment | Cd | | Fe | | Mn | | Zn | | Cu | |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | 0.0 | e | 29.6 | c | 423.9 | a | 244.6 | a | 31.9 | ab |
| *pre*-GSH+Cd | 76.3 | d | 29.8 | c | 237.3 | cd | 125.7 | bc | 33.3 | a |
| *pre*-GB+Cd | 109.6 | c | 28.7 | c | 209.0 | d | 122.9 | bc | 29.4 | ab |
| *pre*-BRs+Cd | 163.2 | a | 27.9 | c | 258.7 | bc | 117.0 | c | 33.3 | a |
| *pre*-SA+Cd | 112.5 | c | 51.0 | a | 232.7 | cd | 131.5 | bc | 31.4 | ab |
| Cd | 141.3 | b | 38.8 | b | 272.6 | b | 139.0 | b | 26.0 | b |

**Table 2:** Effect of GSH, GB, BRs and SA on metal concentration (mg $kg^{-1}$ DW) in shoots of rice seedlings exposed to Cd stress. Different letter presents significant difference among treatments (P<0.05).

| Treatment | Cd | | Fe | | Mn | | Zn | | Cu | |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | 0.0 | d | 343.5 | b | 120.7 | a | 614.5 | a | 110.8 | a |
| *pre*-GSH+Cd | 473.3 | c | 433.2 | a | 75.7 | b | 361.7 | b | 76.9 | c |
| *pre*-GB+Cd | 638.8 | b | 345.5 | b | 72.2 | b | 420.1 | b | 91.7 | b |
| *pre*-BRs+Cd | 676.5 | a | 269.1 | c | 70.9 | b | 335.5 | b | 71.6 | c |
| *pre*-SA+Cd | 618.4 | b | 426.9 | a | 86.6 | b | 372.1 | b | 91.6 | b |
| Cd | 625.1 | b | 447.7 | a | 108.9 | a | 356.4 | b | 87.6 | b |

**Table 3:** Effect of GSH, GB, BRs and SA on metal concentration (mg kg·1 DW) in roots of rice seedlings exposed to Cd stress. Different letter presents significant difference among treatments (P<0.05).

## Conclusion

Recent years, Cd contamination has become a worldwide environmental issue. In this work, we analyzed the possible role of exogenous GSH, GB, BRs and SA pretreatment in modulation antioxidant defense system and mineral absorption against Cd stress in rice. Cd stress induced plant growth inhibition has been well described by many researchers [28,29]. In the current study, shoot height, root length, shoot fresh weight and plant dry biomass were severely decreased at 50 μM Cd treatment compared to control. GSH is the direct substrate for the synthesis of phytochelatins (PCs). GSH acts as a first defense line against metal toxicity through complexing metals before induced synthesis of PCs reaches to an effective level. PCs could bind and sequestrate Cd in stable complex, and then transport the complex to vacuolar. The roles of GSH and PCs in heavy metal tolerance were well illustrated in Cd-sensitive mutants of Arabidopsis [30]. Over expression of glutathione synthetase enhances Cd tolerance in Indian mustard with superior heavy-metal phyto remediation capacity [15]. Pretreatment of GSH, GB or SA to Cd stress medium effectively alleviated Cd-induced growth inhibition and toxicity in rice seedlings, described as its capability to preventing the inhibition of SPAD value, plant height, shoot/root dry weight and chlorophyll content (Table 1 and Figure 1). And GSH, GB and SA pretreatment significantly reduced shoot Cd concentration. Under abiotic stress, the concentration of GSH, GB and SA would increase, which play important role in mediating plants responses to the stress [19,31,32]. Moreover, GSH, GB and SA pretreatment could significantly alleviate the Cd-induced growth inhibition and decrease the Cd content in plants [5,33,34]. So, the results suggested a practical potential for exogenous GSH, GB and SA application as an intervention strategy in alleviating Cd stress and reducing Cd translocation in rice plants. Although BRs markedly increased dry weight, it increased Cd uptake and translocation. Chlorophyll content could indicate the plant health and was more accurate and sensitive than shoot dry weight and root length [12]. Our previous study suggested that Cd could suppress the chlorophyll synthesis [12,28]. In the present study, Cd-induced chlorophyll synthesis inhibition was markedly reverted and the content was even more than control when rice seedlings were pre-treated with GSH, GB or SA for 24 h. Contrary to these results, BRs pretreatment did not affect the chlorophyll synthesis compared with Cd alone treatment. This is opposite with Hayat et al. [35], who found BRs could enhance the chlorophyll content under Cd stress by foliar spray. The possible reason might be due to the difference of methods and plant species. MDA content is considered to be an indicator of lipid peroxidation [28]. In the present study, Cd stress induced oxidative stress characterized significant increase in MDA content compared with control. More MDA accumulation could account for presence of the poisoning reactive oxygen species (ROS) [28]. Under environmental stress conditions, such as Cd, plants have evolved antioxidant enzymes systems, including SOD and POD that are involved in cellular elimination of ROS [36]. SOD catalyzes the decomposition of $O_2$-radicals to $H_2O_2$ and $O_2$ [37]. POD can convert the $H_2O_2$ to H2O [38]. In our study, Cd alone treatment significantly increased leaf SOD and POD activity but decreased the stem POD activity. There is no difference between control and Cd-treated plants in case of stem SOD and root SOD and POD activity. This was in agreement with results of our previous study except leaf SOD activity which was increase after 5 d Cd treatment [5]. The difference might be related to the plant species, interaction between metal and cultivar, and the environment of plant growth. GSH, GB and SA pretreatment effectively alleviate lipid peroxidation as reduced MDA content in shoots, stems and roots (except roots for GB pretreatment). However, BRs pretreatment did not mitigate MDA overproduction.

Meanwhile, application of GSH, GB, BRs and SA did not affect root SOD and POD activity, but reduced leaf SOD activity, compared with Cd alone. GSH and GB pretreatment also increased Cd-induced decrease in stem SOD activity. GB and BRs significantly decreased leaf POD activity. And GSH significantly elevated the depressed stem POD activity. The results showed that the alleviative effects of GSH, GB and SA on Cd phytotoxicity are partly due to the reduction of MDA accumulation and changes of SOD and POD activities. Exposure to 50 μM Cd caused significant reduction in Mn and Zn in shoots, Zn and Cu in roots, and increase in Fe in shoots/roots, which is similar with our previous studies [10,28]. Therefore, excessive Cd accumulation could affect the uptake and distribution of essential mineral elements in crops, and then caused mineral imbalance and inhibition of plant growth. GSH pretreatment decreased Fe in shoots, Mn in shoots/roots, Cu in roots and increased shoot Cu when compared with Cd alone treatment. GB pretreatment decreased the Fe and Mn in shoots/roots. BRs pretreatment decreased the Fe in shoots/roots, Mn and Cu in roots, Zn in shoots, and increased Cu in shoots. And SA pretreatment elevated the Fe in shoots and decreased Mn in shoots/roots. Thus, the varying uptake and distribution of Fe, Mn, Zn and Cu may involve in the plant tolerance against Cd stress. In conclusion, 24 h pretreatment of GSH, GB and SA significant alleviated Cd-induced inhibition on growth and chlorophyll content, reduced shoot Cd concentration and markedly diminished Cd-induced MDA accumulation. GSH, GB and SA pretreatments counteracted the pattern of alterations in certain antioxidant enzymes induced by Cd, e.g. suppressed Cd-induced dramatic increase of leaf SOD activity, GB and BRs also significantly decreased leaf POD activity; GSH significantly elevated the depressed stem POD and SOD activities; and GB elevated stem SOD activity. GSH and GB also counteracted Cd-induced response of element concentration: GSH suppressed Cd-induced dramatic increase of leaf Fe and elevating Cd-depressed leaf Cu; GB decreased Cd-induced increase in root/leaf Fe. However, BRs pretreatment may enhance Cd uptake and translation from root to shoot, accordingly, BRs would be unsuitable for the edible crops grown in Cd contaminated soils to alleviate phyto-toxicity of Cd, although the root length and plant dry weight were increased over Cd alone treatment.

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