



AsA–GSH Cycle and Antioxidant Enzymes Play Important Roles in Cd Tolerance of Wheat

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Abstract

Wheat (*Triticum aestivum* L.) has relatively high tolerance to cadmium (Cd), but the underlying mechanisms are poorly understood. Growth and physiological parameters of wheat exposed to different Cd concentrations (0, 0.5, 5 and 50 μM) were characterized. The fresh weight, leaf chlorophyll and carotenoid concentrations and photosynthesis parameters did not differ among Cd treatments, suggesting relatively high Cd tolerance in wheat. However, the soluble sugar concentrations increased with the increasing Cd concentration and the soluble protein concentrations decreased in both shoots and roots, suggesting that the Cd application promoted nitrogen metabolism over carbon metabolism. In addition, the higher concentrations of MDA, GSH and AsA and activities of antioxidant enzymes (SOD, POD, and CAT) were observed in leaves and roots in the Cd50 treatment. Our results reveal that wheat can tolerate Cd by enhancing the antioxidant enzymes activities and increasing the concentration of ascorbate and glutathione.

Keywords Cadmium · Wheat · Photosynthesis · Ascorbate · Glutathione · Antioxidant enzymes

During the last few decades, heavy metal contamination, especially for cadmium (Cd), in the environment has been increasingly severe, causing toxicity and diminished output of crops (Rizwan et al. 2016a). Under Cd stress, the initial symptom in plants is usually chlorosis, suggesting the photosynthetic apparatus is particularly susceptible to Cd and a reduction of photosynthesis is a common response in plants (Burzyński and Kłobus 2004). In addition to photosynthesis, Cd toxicity might also alter a battery of physiological and biochemical processes including seed germination, gas exchange, stem and root growth, enzyme activity, hormonal balance, nutrient assimilation, protein synthesis, and DNA replication, with a resultant decrease in biomass production (Gallego et al. 2012). Cd exerts toxicity through four proposed mechanisms, including (i) competing for cation absorption, (ii) disrupting the structure and function of proteins, (iii) displacing essential cations from specific binding sites, and (iv) enhancing generation the reactive oxygen species (ROS) (Dalcorso et al. 2013). However, plants can

resist Cd toxicity by a range of mechanisms, such as reducing Cd uptake and root to shoot translocation, and detoxifying/sequestering Cd (Singh et al. 2016). Moreover, the plant antioxidant defense system plays a crucial role in combating the Cd damage. Antioxidants comprise ROS-removing enzymes such as superoxide dismutase (SOD), catalases (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), and non-enzymatic systems such as ascorbic acid (ASA) and glutathione (GSH) (Shah et al. 2001; Thounaojam et al. 2012). SOD is an enzyme involved in dismutating superoxide radicals in all the cellular compartments (Fridovich 1989). APX is located in the cytosol and chloroplast and is involved in scavenging of H_2O_2 (Willekens et al. 1997) CAT is an oxidoreductase enzyme catalyzing decomposition of H_2O_2 into H_2O and O_2 during photorespiration (Morita et al. 1994). AsA and GSH are hydroxyl radical scavengers and may also chelate Cd in the cytoplasm (Wu et al. 2015).

Wheat (*Triticum aestivum*) is a globally important crop being the staple food for humans and livestock. Cd is highly toxic to wheat (Jin et al. 2010; Rizwan et al. 2016a). The objectives of the present study were to characterize the responses of antioxidant enzyme and the ascorbate–glutathione cycle to Cd stress in wheat. The results from our study will improve the understanding of Cd tolerance

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mechanisms in wheat and help develop strategies for alleviating Cd toxicity in wheat cultivation.

Materials and Methods

Zhengmai 379, a commonly-grown cultivar in the Huanghuai winter wheat area in China, was used in this experiment. A hydroponic experiment was conducted in a controlled environment chamber and seed germination and cultivation were as described in our previous study (Qin et al. 2017b). The nutrient solution contained 0.5 mM KH_2PO_4 , 0.5 mM K_2HPO_4 , 0.5 mM $\text{Mg}(\text{NO}_3)_2 \cdot 7\text{H}_2\text{O}$, 1 mM KNO_3 , 1.0 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.125 mM KCl , 50 μM H_3BO_3 , 12 μM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.7 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 100 μM FeNaEDTA and 0.25 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. Twelve seedlings of uniform size were cultured in a plastic container filled with 2 L of nutrient solution. Cd was applied as CdCl_2 at four final concentrations: 0 (control), 0.5 μM (Cd0.5), 5 μM (Cd5) and 50 μM (Cd50). Each treatment was replicated four times. The nutrient solutions were renewed every 3 days and adjusted to maintain pH at 6.5 ± 0.1 with HCl or NaOH . After 25 days of treatment, plants were harvested and divided into roots and shoots, and oven dried or stored fresh at -80°C for further analyses.

Approximately 300 mg of each sample was digested with a mixture of 6 mL of nitric acid:perchloric acid (4:1, v/v). Cd was measured by flame atomic absorption spectrometry (ZEE nit 700, Analytik Jena AG, Germany) as described by Xin et al. (2016). Chlorophyll and carotenoid contents were determined by a spectrophotometer (TU-1901, Beijing, China). Just before harvest, net photosynthetic rate (P_n), stomatal conductance (G_s), transpiration rate (T_r) and intercellular CO_2 concentration (C_i) were measured by a portable photosynthesis system (Li-6400P, Li-Cor, USA) according to Qin et al. (2017a).

The extent of lipid peroxidation was estimated by determining malondialdehyde (MDA) formation using the

thiobarbituric acid (TBA) method (Ci et al. 2009). Briefly, 0.5 g of fresh shoot or root samples was homogenized with 2 mL of 5% v/v trichloroacetic acid (TCA) and centrifuged at $10,000 \times g$ for 10 min. Then, 1 mL 0.5% v/v TBA in 20% v/v TCA was added to 1 mL supernatant and incubated in boiling water bath for 30 min, followed by an immediate cooling on ice to stop the reaction. Thereafter, the mixture was centrifuged at $10,000 \times g$ for 5 min. The absorbance was determined at 450, 532 and 600 nm, and MDA concentration was estimated by the formula: $\text{MDA} (\mu\text{mol/g FW}) = 6.45 (\text{OD}_{532} - \text{OD}_{600}) - 0.56 \text{OD}_{450}$.

Soluble sugars and soluble protein in root and leaf tissues were assayed as described by Qin et al. (2017a). GSH was assayed by an enzyme recycling procedure (Anderson 1985). Fresh shoot or root samples (0.5 g) were homogenized in 5 mL acidic extraction buffer [5% w/v potassium-phosphoric acid in 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA)]. The mixture was centrifuged at $14,000 \times g$ for 10 min. The supernatant was collected for analysis of GSH at 412 nm using a spectrophotometer (TU-1901, Beijing, China). Results were expressed as $\mu\text{mol GSH g}^{-1}$ fresh weight.

Antioxidant enzyme activities (SOD, CAT and POD) in roots and leaves were determined spectrophotometrically. Fresh tissues (0.5 g) were ground in liquid nitrogen to a fine powder using a mortar and pestle. The powder was transferred to a pre-cooled (4°C) mortar and pestle with 2 mL of 50 mmol L^{-1} phosphate buffer (pH 7.0), containing 0.1 mmol L^{-1} EDTA, 4% (w/v) polyvinylpyrrolidone and 0.3% v/v Triton X-100. The homogenate was centrifuged at $15,000 \times g$ AT 4°C for 20 min. The supernatant was used for assays of the antioxidant enzyme activities (Beauchamp and Fridovich 1971; Aebi 1984; Zhang et al. 2012).

All data were statistically analyzed using SPSS 20.0 software. Statistical evaluation was performed with ANOVA (LSD-test, $p \leq 0.05$), and the results were expressed as the mean values \pm SE (standard error) of four biologic replicates.

Table 1 Biomass and Cd concentration and content in wheat grown for 25 days with various concentrations of Cd

Index	Cd0	Cd0.5	Cd5	Cd50
Total plant FW (g)	2.64 \pm 0.09a	2.61 \pm 0.20a	2.53 \pm 0.13a	2.21 \pm 0.17a
Shoot FW (g plant ⁻¹)	1.80 \pm 0.10a	1.88 \pm 0.15a	1.80 \pm 0.10a	1.57 \pm 0.12a
Root FW (g plant ⁻¹)	0.84 \pm 0.02a	0.73 \pm 0.07ab	0.73 \pm 0.04ab	0.64 \pm 0.05b
Shoot Cd concentration ($\mu\text{g g}^{-1}$)	ND	3.31 \pm 0.40c	19.5 \pm 1.56b	71.0 \pm 0.92a
Root Cd concentration ($\mu\text{g g}^{-1}$)	ND	24.7 \pm 2.18c	159 \pm 6.01b	352 \pm 10.6a
Shoot Cd content ($\mu\text{g plant}^{-1}$)	ND	0.94 \pm 0.10c	5.68 \pm 0.58b	19.5 \pm 1.74a
Root Cd content ($\mu\text{g plant}^{-1}$)	ND	2.06 \pm 0.19c	13.7 \pm 0.82b	28.7 \pm 1.57a

Mean \pm SE, n=4

For each parameter, different letters indicate significant differences among the four treatments at $p < 0.05$ as determined by LSD test

ND not detected

Results and Discussion

Wheat shoot FW was not significantly affected by the Cd treatments (Table 1), but root FW decreased significantly by 24% at the Cd50 treatment compared with the Cd0 treatment. As expected, the Cd concentration and content in shoots and roots increased significantly by the Cd treatment. For the increasing Cd application rates, the Cd concentrations were

7.5, 8.2 and 5.0-fold higher in roots than shoots, and the Cd contents were 2.2, 2.4 and 1.5-fold higher in roots than shoots.

As reported previously, soil addition of Cd affected the seedling growth of wheat, even at low Cd rates ($< 3.3 \text{ mg kg}^{-1}$) (Lin et al. 2007). Naem et al. (2016) also reported that root and shoot relative dry matter decreased in most wheat cultivars, but some cultivars did not exhibit any

Table 2 The leaf photosynthetic pigment concentrations and the photosynthetic parameters of wheat grown for 25 days in different Cd treatments

Treatment	Chlorophyll a (mg g^{-1})	Chlorophyll b (mg g^{-1})	Carotenoid (mg g^{-1})	P_n ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	G_s ($\text{mol m}^{-2} \text{ s}^{-1}$)	C_i ($\mu\text{mol mol}^{-1}$)	T_r ($\text{mol m}^{-2} \text{ s}^{-1}$)
Cd0	$2.36 \pm 0.05\text{a}$	$0.95 \pm 0.04\text{a}$	$0.32 \pm 0.00\text{a}$	$32.0 \pm 2.41\text{a}$	$0.22 \pm 0.03\text{ab}$	$492 \pm 50.6\text{ab}$	$4.43 \pm 0.47\text{ab}$
Cd0.5	$2.20 \pm 0.04\text{a}$	$0.84 \pm 0.02\text{a}$	$0.31 \pm 0.00\text{a}$	$35.3 \pm 1.32\text{a}$	$0.27 \pm 0.06\text{a}$	$554 \pm 38.7\text{a}$	$5.36 \pm 0.74\text{a}$
Cd5	$2.19 \pm 0.10\text{a}$	$0.82 \pm 0.06\text{a}$	$0.30 \pm 0.01\text{ab}$	$33.3 \pm 1.13\text{a}$	$0.15 \pm 0.01\text{b}$	$478 \pm 12.6\text{ab}$	$3.61 \pm 0.10\text{b}$
Cd50	$2.16 \pm 0.12\text{a}$	$0.85 \pm 0.08\text{a}$	$0.28 \pm 0.00\text{b}$	$33.6 \pm 1.51\text{a}$	$0.21 \pm 0.04\text{ab}$	$422 \pm 64.1\text{b}$	$4.46 \pm 0.58\text{ab}$

Means \pm SE, $n=4$

For each parameter, different letters indicate significant differences among the four treatments at $p < 0.05$ as determined by LSD test

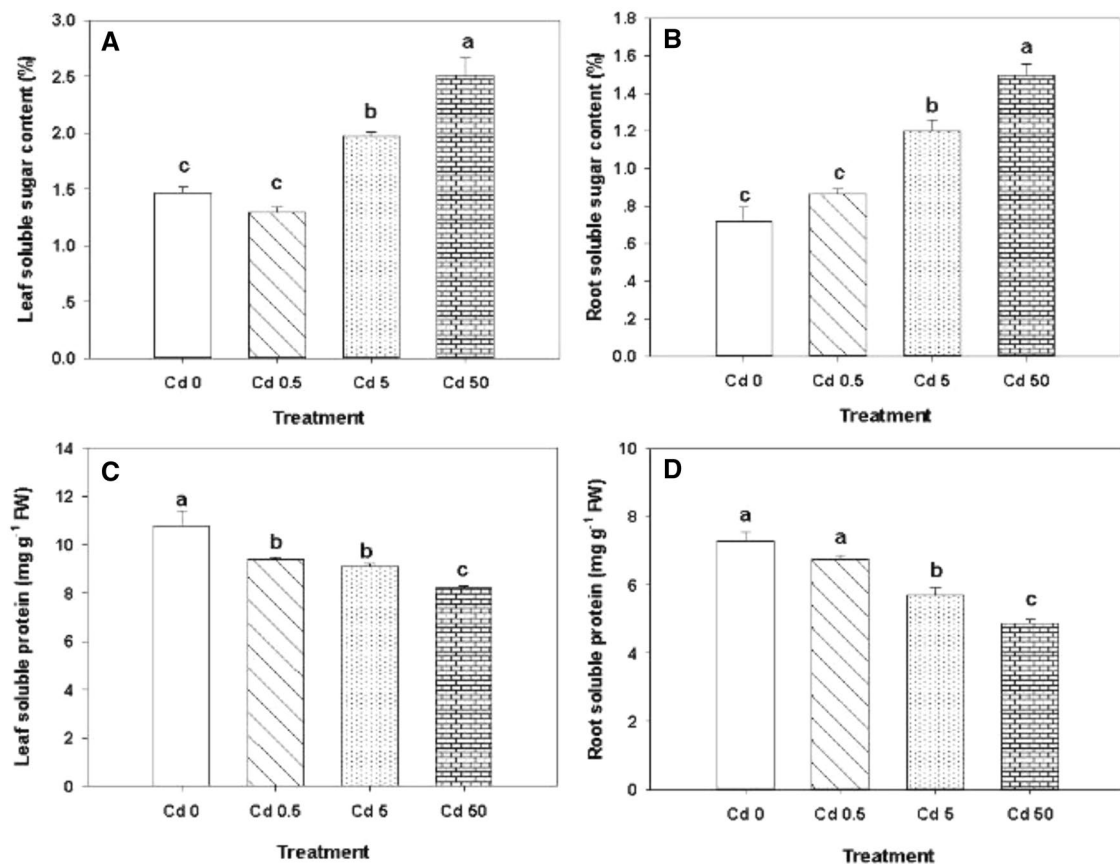


Fig. 1 Concentration of soluble sugars (a: leaves, b: roots) and soluble proteins (c: leaves, d: roots) in winter wheat grown for 25 days under different Cd concentration treatments. Means (\pm SE), $n=4$.

Bars with different letters are significantly different at $p < 0.05$ (LSD's test). The same applied for Figs. 2 and 3

toxic symptoms, even at 45 μM Cd concentration. These findings indicated that the wheat cultivar used in the study reported here was relatively tolerant to Cd, with some toxicity obvious in roots only.

Cd did not significantly influence concentration of chlorophyll a and b, but carotenoids concentration was lower in the Cd50 compared with C0 and Cd0.5 treatments (Table 2). There was no significant difference in leaf P_n among the four

Cd treatments, and no consistent pattern of the Cd effect on G_s , C_i and T_r .

Compared with the Cd0 treatment, the leaf soluble sugar concentrations increased significantly in the treatments Cd5 (by 34%) and Cd50 (by 72%) (Fig. 1a), and the root soluble sugar concentrations increased significantly by 67% and 108% at Cd5 and Cd50, respectively (Fig. 1b). Similarly, Costa and Spitz (1997) reported that the soluble

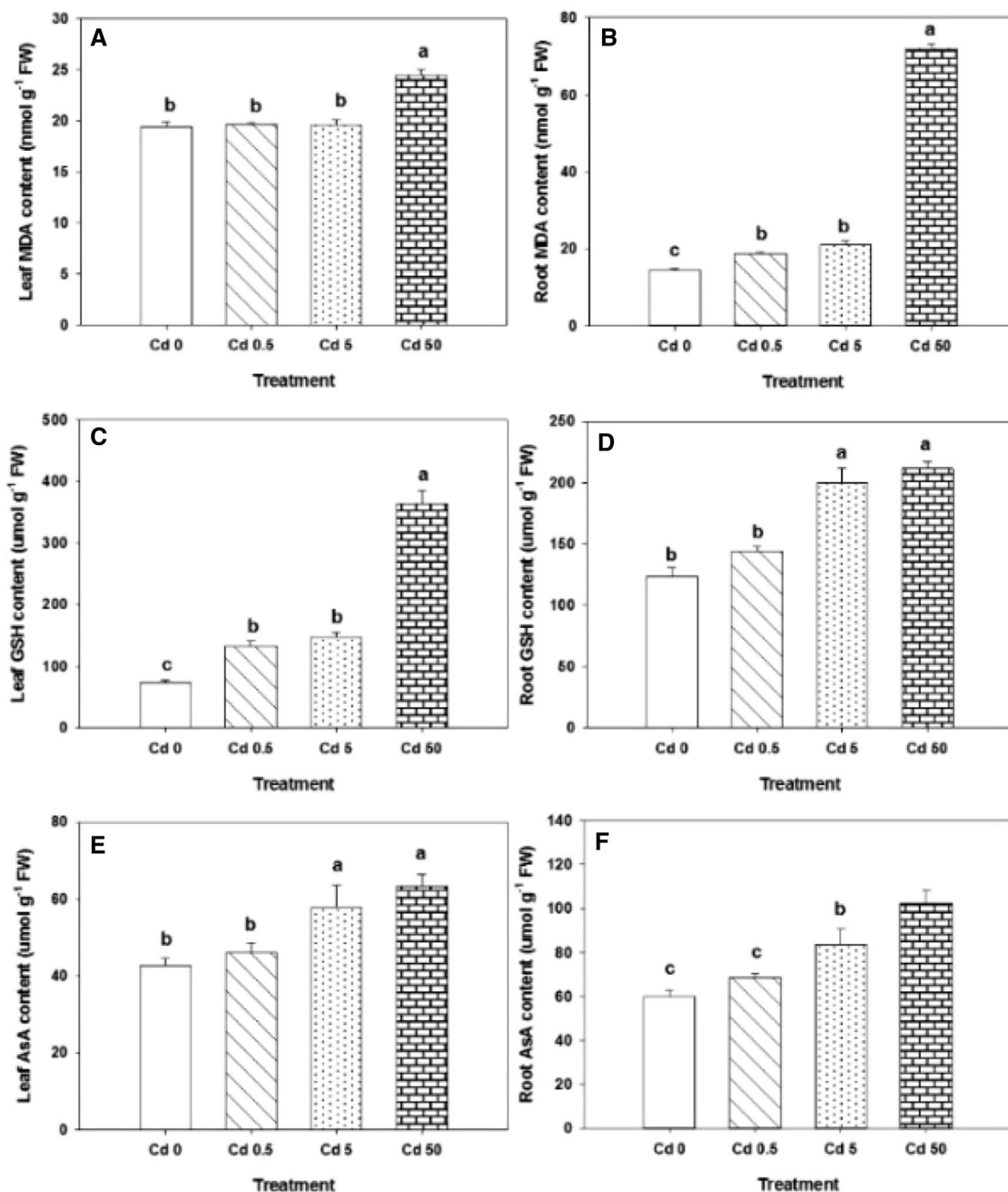


Fig. 2 Concentration of MDA concentration (a, b), GSH (c, d), and AsA (e, f) in leaves (a, c, e) and roots (b, d, f) of winter wheat under different Cd concentration treatments

carbohydrate concentrations, ragginose and mannose in *Lupinus albus* increased with increasing Cd concentrations. Compared with the Cd0, the leaf soluble protein concentrations decreased significantly in the treatments Cd5 (by 12%) and Cd50 (by 20%) (Fig. 1c), and the root soluble sugar concentrations decreased significantly by 22% and 33% at Cd5 and Cd50, respectively (Fig. 1d). These results suggest a nutritional imbalance between proteins and sugars

in Cd-treated plants (Gutiérrez et al. 2007; Costa and Spitz 1997).

The Cd50 treatment was associated with increases in MDA concentration of 26% in leaves ($p < 0.05$) (Fig. 2a) and 397% in roots ($p < 0.05$) (Fig. 2b) compared with the Cd0 treatment (Fig. 2b). These findings were in agreement with the other reports on the Cd effects in wheat (Sun et al. 2007; Wu et al. 2015), indicating that Cd toxicity was severe

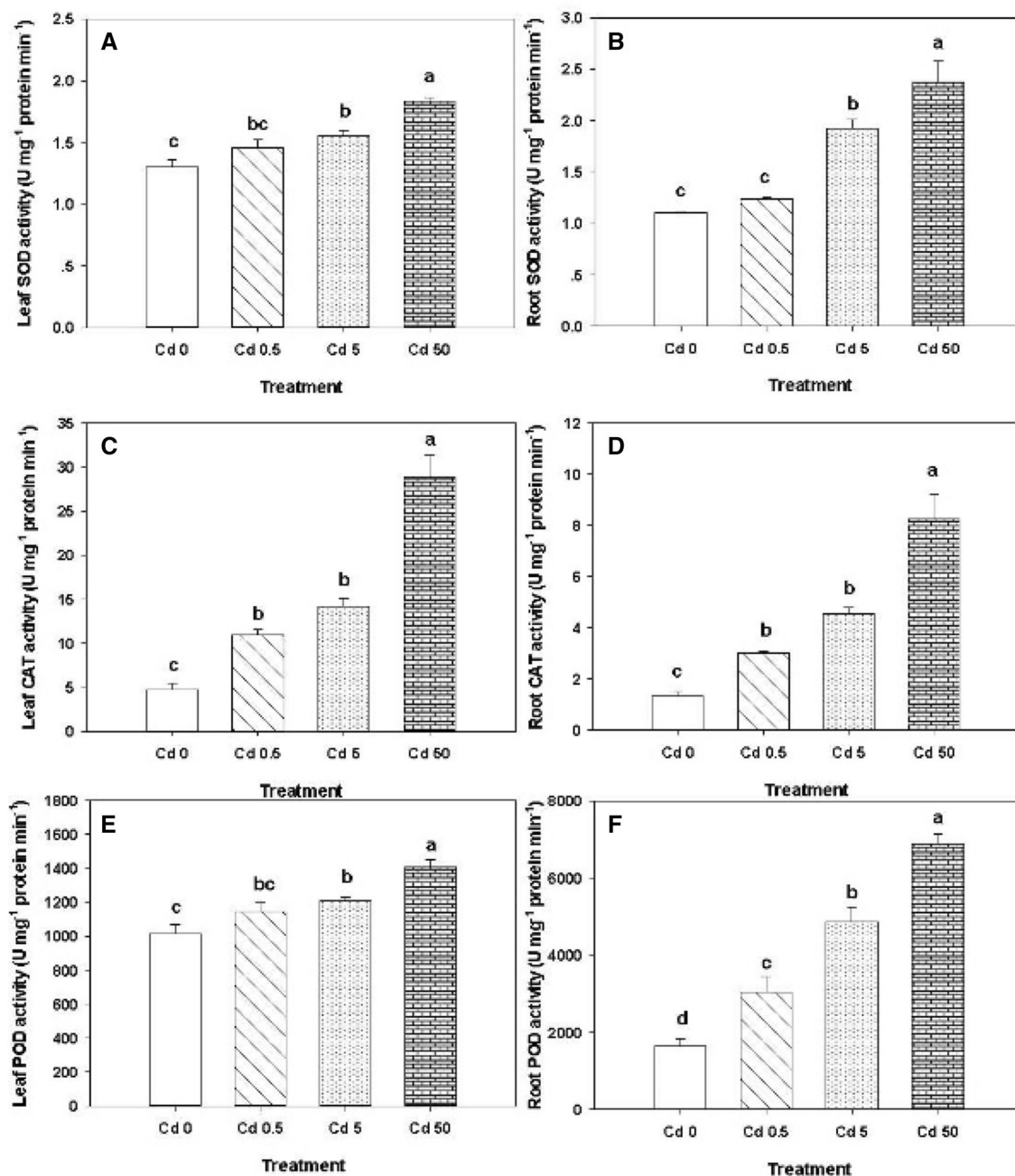


Fig. 3 Activities of SOD (a, b), CAT (c, d) and APX (e, f) in leaves (a, c, e) and roots (b, d, f) of winter wheat grown for 25 days under different Cd concentration treatments

in root. The treatments Cd0.5, Cd5 and Cd50 increased GSH concentration significantly (by 84%, 103% and 401% respectively) ($p < 0.05$), compared with Cd0 (Fig. 2c). In roots, there were no significant differences in GSH concentration between Cd0 and Cd0.5 (Fig. 2d). Cd5 and Cd50 treatments increased GSH concentration significantly [by 62% and 71% respectively ($p < 0.05$)], compared with the Cd0 treatment (Fig. 2d). The AsA concentration in leaves (Fig. 2e) and roots (Fig. 2f) increased gradually with increasing Cd concentration in nutrient solution, and the highest leaf and root AsA concentrations were recorded in Cd50 treatment. The Cd5 and Cd50 treatments were associated with significantly ($p < 0.05$) increased AsA concentrations in leaves (by 36% and 49%, respectively), compared with Cd0 (Fig. 2f). As reported, Cd-tolerant genotypes had higher concentrations of GSH and AsA than Cd-sensitive genotypes (Wang et al. 2011; Wu et al. 2015). These results suggested that increasing GSH and AsA concentrations may protect wheat from increasing concentration of Cd (Wu et al. 2015; Rizwan et al. 2016a).

Another important strategy to reduce Cd stress is enhancing the antioxidative enzymes, including SOD, CAT and APX (Ci et al. 2009; Rizwan et al. 2016b). In the study presented here, SOD activities increased significantly ($p < 0.05$) in the Cd5 (by 19% in leaves and 74% in roots) and Cd50 treatments (by 41% in leaves and 115% in roots) compared with the control (Cd0) (Fig. 3a, b). In the Cd0.5, Cd5 and Cd50 treatments, CAT activities in leaves increased by 131%, 198% and 507%, respectively (Fig. 3c), and in roots by 122%, 235% and 511%, respectively, compared with Cd0

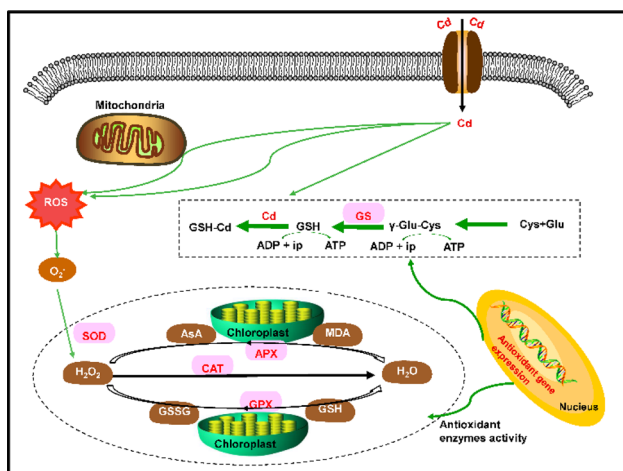


Fig. 4 Cd exposure induced-oxidative stress that activated the tolerance and detoxification mechanisms in wheat cells according to the description of Singh et al. (2016). *Glu* glutamine, *Cys* cysteine, *AsA* ascorbic acid, *GSSG* oxidized glutathione, *Gly* glycine, H_2O_2 hydrogen peroxide, O_2^- superoxide radicals, *ROS* reactive oxygen species, *GS* glutathione synthetase, *GPX* glutathione peroxidase, *APX* ascorbate peroxidase

treatment (Fig. 3d). The APX activities in leaves and roots also increased with an increase in the Cd application. Compared with the C0 control, in the Cd5 and Cd50 treatments APX activities in leaves increased by 19% and 38% respectively (Fig. 3e), and in roots by 195% and 318%, respectively (Fig. 3f). These results indicated that the antioxidant system varied among wheat genotypes to reply the Cd tolerance and was also demonstrated to increase with increasing Cd supply (Fig. 4), suggesting that the antioxidant system in wheat was activated by Cd application in adose-dependent manner. Furthermore, it also demonstrated that antioxidant enzyme activities may contribute to Cd tolerance in wheat.

In conclusion, different wheat varieties have different Cd tolerance strategies. Our results suggest that the wheat used in our experiment had considerable tolerances to Cd. The tolerance mechanism can be explained by the high-efficiency in AsA–GSH cycle and antioxidant enzyme activities. Further studies focusing on the molecular mechanisms are still necessary.

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