Cadmium-induced oxidative stress and protection by L-Galactono-1, 4-lactone in winter wheat (*Triticum aestivum L.*)

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Summary

Two hydroponic culture experiments were conducted to investigate cadmium (Cd)-induced oxidative stress in winter wheat (*Triticum aestivum* L.) seedlings and the effects of L-Galactono-1, 4-lactone (GalL), the biosynthetic precursor of the antioxidant ascorbate (AsA), on the oxidative stress induced by Cd. In experiment 1, with application of Cd (0, 10, 25, 50 μM) in nutrient solution, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) levels as well as membrane permeability in both shoots and roots were significantly increased, indicating Cd-induced oxidative stress and lipid peroxidation as well as plasma-membrane damage in the plants. In experiment 2, H_2O_2 levels in plants exposed to Cd were significantly reduced by the addition of GalL (25 mM), associated with increased activities of peroxidase (POD), indicating that GalL alleviated the oxidative stress induced by Cd. Unexpectedly, however, the MDA levels were not reduced by the addition of GalL. Does Cd also induce lipid peroxidation directly besides *via* formation of reactive oxygen species (ROS)? This needs further study.

Key words: cadmium / oxidative stress / winter wheat / L-Galactono-1, 4-lactone

1 Introduction

Cadmium (Cd) contamination is wide-spread over the world. Anthropogenic emissions of Cd are estimated to be about 30,000 t annually (*di Toppi* et al., 1998), which resulted in increased levels of Cd in agricultural soils and consequently in food crops. Cadmium contamination of food poses a great danger for human health such as renal dysfunction, liver damage, lung edema, anemia, and hypertension (*Basta* et al., 1998). Cadmium has no known biological functions and is highly toxic to animals and plants. Even lower levels of Cd were found to induce oxidative stress in cells, commonly accompanied by an accumulation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$) (*Somashekaraiah* et al., 1992; *Milone* et al., 2003), whose levels should be kept as low as possible in order to avoid damage to cells (*Schützendübel* and *Polle*, 2002). Control of oxidant levels is achieved by a complex antioxidative system that includes enzymatic and nonenzymatic components. Antioxidant enzymes include catalase (CAT, E.C.1.11.1.6.), guaiacol peroxidase (POD, E.C.1.11.1.7), ascorbate peroxidase (APX, E.C.1.11.1.11), superoxide dismutase (SOD, E.C.1.15.1.1), glutathione reductase (E.C.1.6.4.2), dehydroascorbate reductase (DAR, E.C.1.8.5.1), monodehydroascorbate radical reductase (MDAR, E.C.1.6.5.4), and the nonenzymatic mechanisms consist of radical-scavenging compounds such as ascorbate (AsA) and glutathione (GSH) (*Schützendübel* et al., 2002). There is ample evidence that Cd exposure can result in changes of antioxidant levels and activities of antioxidant enzymes in plants (*Shaw*, 1995; *Luo* et al., 1998; *Schickler* and *Caspi*, 1999; *Hegedüs* et al., 2001; *Schützendübel* et al., 2001; *Olmos* et al., 2003). AsA is a powerful antioxidant, it functions together with GSH and other antioxidant enzymes to constitute a GSH–AsA cycle by which mainly H_2O_2 is detoxified. Several reports have shown the role of AsA in alleviating ozone toxicity by detoxifying ROS (*Mächler* et al., 1995; *Moldau* et al., 1998; *Zheng* et al., 2000). There is evidence that L-Galactono-1, 4-lactone (GalL) is the biosynthetic precursor of the antioxidant AsA (*Wheeler* et al., 1998). Plants treated with GalL showed enhanced tolerance to ozone (*Maddison* et al., 2002). The aim of the present study was to investigate the alleviating effect of GalL on the oxidative stress in plants induced by Cd.

2 Materials and methods

2.1 Experiment 1: oxidative-stress toxicity of Cd

2.1.1 Plant culture and Cd treatment

Seeds of winter wheat (*Triticum aestivum* L., cv. Yuandong 977) were surface-sterilized in 10% H_2O_2 for 10 min followed by thorough washing in deionized water, then germinated on moist filter paper for 2 d. Germinated seeds were transferred to moist perlite and cultivated for about 1 week. The seedlings were then washed carefully under tap water and transferred to PVC pots containing 1100 mL modified Hoaglands nutrient solution containing (in mM:) KNO_3, 1.33; $Ca(NO_3)_2$, 1.33; $MgSO_4$, 0.5; KH_2PO_4, 0.44; (in μM:) FeEDTA, 50.0; $CuSO_4$, 0.5; MnSO4, 2.5; H_3BO_3, 5; Na_2MoO_4, 0.25; $CoSO_4$, 0.09; NaCl, 50.0; $ZnSO_4$, 1.0. The solution was treated with different $CdCl_2$ concentrations (0, 10, 25, 50 μM). Each treatment had four replicates. The seedlings were grown in a growth chamber with 14/10 h light/dark cycles. Light intensity was around 280 μmol photons m^{-2} s^{-1}. The nutrient solution was renewed twice a week and aerated continuously. Pots were randomly rearranged every day during the growth period of 10 d.

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2.1.2 Histochemical staining of roots for hydrogen peroxide H_2O_2

To detect H_2O_2 directly, fresh roots were stained following the method of *Schützendübel* et al. (2002). Fresh roots attached to the plants were stained in KI/starch reagent containing 0.1 M KI (pH 5.0) and 4% starch (w/v) for 30 min. Stained roots were photographed under a binocular (XSZ-H, Tike, China).

2.1.3 Membrane-permeability measurement (K^+ efflux)

Membrane permeability was measured according to *Llamas* et al. (2000) with minor modifications. At harvest, seedling roots of each treatment were washed twice with cold 1 mM $CaCl_2$ to eliminate nutrient solution from the apoplast and then blotted with filter paper. Washed roots of each treatment were transferred to 25 mL fresh $CaCl_2$ solution for 4 h with continuous aeration in a growth chamber. After 4 h, the K^+ content of the solution was measured by ICP–OES (Inductively Coupled Plasma–Optical Emission Spectrometry, Optima 2000 DV, PerkinElmer, USA). Membrane permeability was expressed as K^+ efflux per gram of fresh roots (μmol K^+ (g FW)$^{-1}$).

2.2 Experiment 2: Effects of GalL on Cd-toxicity–induced oxidative stress

2.2.1 Plant culture, GalL and Cd treatments

Plant culture was as described in experiment 1. When transferred to PVC pots, two-thirds of the seedlings were treated with 25 mM L-Galactono-1, 4-lactone (+GalL) for 24 h. Then half of the seedlings were transferred into a solution supplemented with 25μM $CdCl_2$ (+Cd), 3 d later, half of the +GalL seedlings were treated with 25 mM L-Galactono-1, 4-lactone for 24 h again (++GalL). The control seedlings grew in standard nutrient solution with no Cd and GalL addition. All together, there were six treatments as shown in Tab. 1. Plants were harvested after 6 days of treatment.

Table 1: Experimental treatments.

1	2	3	4	5	6
Control (–GalL –Cd)	–GalL +Cd	+GalL –Cd	+GalL +Cd	++GalL –Cd	++GalL +Cd

2.2.2 Quantitative analysis of H_2O_2

Fresh plant material (2 g) was homogenized in cooled acetone (2 mL) and the resulting slurry centrifuged at 10,000 *g* for 10 min. One milliliter of the supernatant was mixed with 0.1 mL 5% $Ti(SO_4)_2$ and 0.2 mL ammonia. After the precipitate was formed, the reaction mixture was centrifuged at 10,000 *g* for 10 min. The resulting pellet was dissolved in 2 M H_2SO_4, and the absorbance was read at 415 nm (*Mukherjee* and *Choudhuri*, 1983).

2.2.3 Analysis of lipid peroxidation

The level of lipid peroxidation was determined as malondialdehyde (MDA) content and was measured according to *Heath* and *Packer* (1968). About 200 mg fresh plant material was homogenized with 10 mL 0.25% 2-thiobarbituric acid (TBA) in 10% trichloro-acetic acid (TCA). The homogenate was incubated at 95 °C for 30 min followed by rapid cooling in an ice bath and centrifugation at 10,000 *g* for 10 min. The absorbance of the resulting supernatant was read at 532 nm and the nonspecific absorbance at 600 nm was subtracted. MDA contents were calculated using the absorption coefficient of MDA (ε = 155 mmol L^{-1} cm^{-1}).

2.2.4 POD extraction and assay

Frozen plant leaves or roots (1 g) were homogenized in 5 mL 50 mM phosphate buffer (pH 7.0) containing 1% (w/v) polyvinylpyrolidone (PVP) and centrifuged at 10,000 *g* for 20 min. All steps were carried out at 4 °C. The supernatant was used to assay the activity of POD. The protein concentration of the supernatant was determined by comparing absorptions at 280 and 260 nm, using an UV/Vis Spectrophotometer (UV-3100, Hitachi Co., Japan). POD activity was assayed according to *Mazhoudi* et al. (1997). Enzyme activity was expressed as increase of absorption units per min and mg protein (U min^{-1} (mg protein)$^{-1}$).

2.3 Data analysis

All data were subjected to analysis of variance (ANOVA) using commercially available GENSTAT (6th ed., NAG Ltd, England).

3 Results

3.1 Cd-induced oxidative stress in plants

3.1.1 H_2O_2 accumulation

The extent of root staining was dependent on the Cd concentration in the nutrient solution (Fig. 1). Thus, the roots at 50 μM had the deepest color, which suggested that Cd induced H_2O_2 accumulation. The results of the quantitative analysis of H_2O_2, as shown in Fig. 2, were in agreement with the staining results. Contents of H_2O_2 in shoots and roots increased significantly with increasing Cd concentrations ($p < 0.001$). Compared to the control, H_2O_2 levels at 25 μM were increased about 5-fold in both shoots and roots, and 6.5-fold in shoots and even 20-fold in roots at 50 μM. Hydrogen peroxide is an important reactive oxygen species, and its level is one of the most important indicators for oxidation. Both the staining and the quantitative analysis indicated that Cd induced oxidative stress in plants.

3.1.2 Membrane permeability

Membrane permeability is an indicator for the extent of damage of membrane structure and is usually expressed as K^+ efflux. Figure 3 shows that K^+ efflux was dependent on the Cd concentration. Addition of Cd induced pronounced increase in K^+ efflux, indicating Cd-induced damage of membrane structure.

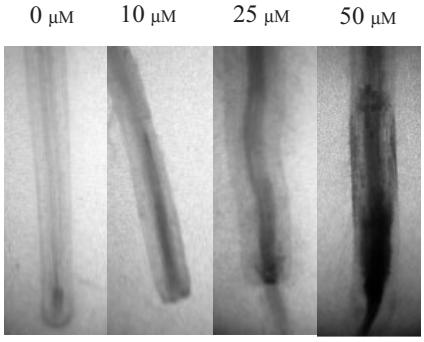

Figure 1: H_2O_2 accumulation in roots of wheat seedlings grown in nutrient solution treated with different Cd concentrations (0, 10, 25, 50 μM).

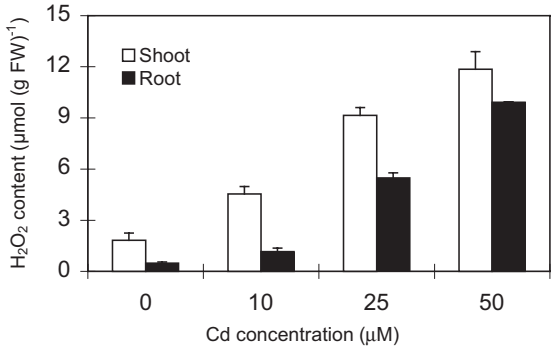

Figure 2: H_2O_2 contents in shoots and roots of winter wheat seedlings treated with different Cd concentrations. Values are means ± S.D. of four replications.

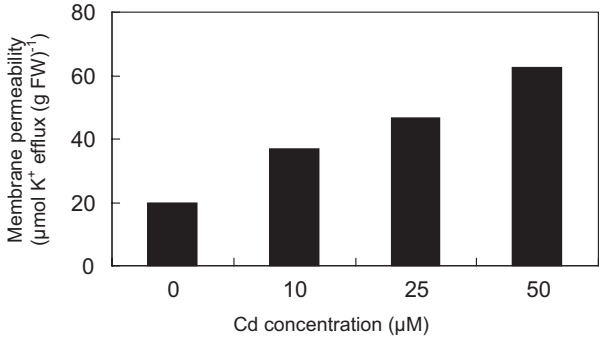

Figure 3: Root-cell–membrane permeability of winter wheat seedlings treated with different Cd concentrations. Measurement of K^+ in a $CaCl_2$ solution after a 4 h efflux period.

3.1.3 Lipid peroxidation

MDA concentrations in shoots and roots increased significantly ($p < 0.001$) with increasing Cd concentrations in the nutrient solution (Fig. 4). In shoots, MDA concentration was about 3-fold higher at 50 μM in comparison to the control. The effect on the roots was even more pronounced, an increase in Cd supply from 0 to 10, 25, and 50 μM led to an increase in MDA concentrations from 2.53 nmol (g FW)$^{-1}$ to 7.32 nmol (g FW)$^{-1}$, 9.79 nmol (g FW)$^{-1}$, and 10.81 nmol

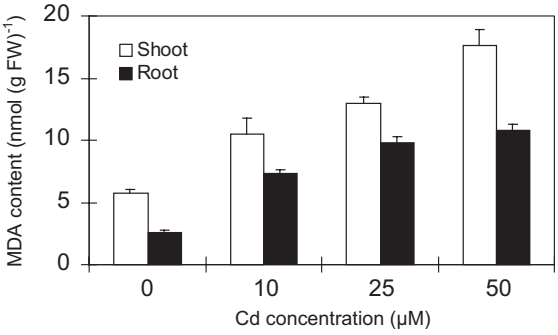

Figure 4: MDA contents in shoots and roots of winter wheat seedlings treated with different Cd concentrations. Values are means ± S.D. of four replications.

(g FW)$^{-1}$. The results were in agreement with results of H_2O_2 formation and membrane permeability.

3.2 Effects of GalL on Cd-induced oxidative stress in plants

3.2.1 Effects of GalL on H_2O_2 accumulation

Under condition of no Cd stress (–Cd), addition of GalL (+GalL) had no significant effect on the accumulation of H_2O_2 in plants compared to non-GalL addition (–GalL) (Fig. 5). Under Cd stress (+Cd), however, +Cd increased H_2O_2 level for about 56% compared to the control, indicating accumulation of H_2O_2 induced by Cd. Addition of GalL pronouncedly decreased H_2O_2 content from 11.4 μmol (g FW)$^{-1}$ of –GalL+Cd to 4.5 μmol (g FW)$^{-1}$. GalL addition for a second time in comparison to one addition showed no pronounced effect on H_2O_2 accumulation.

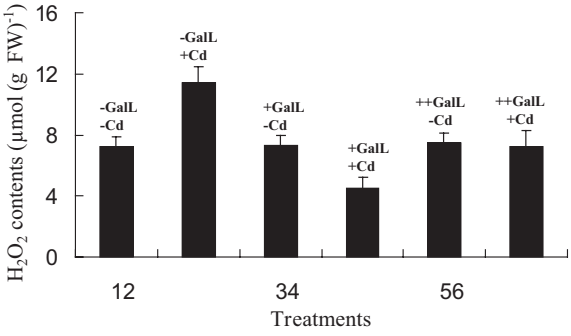

Figure 5: H_2O_2 contents in shoots of winter wheat seedlings grown in nutrient solution with different GalL (0, 25 mM) and Cd (0, 10, 25, 50 μM) treatments. Values are means ± S.D. of four replications.

3.2.2 Effects of GalL on lipid peroxidation

In agreement with experiment 1, MDA contents in shoots of +Cd treatment were all higher than in –Cd treatment (Fig. 6). With respect to GalL treatment, however, GalL addition neither for the first time nor for the second time had a significant effect on MDA contents.

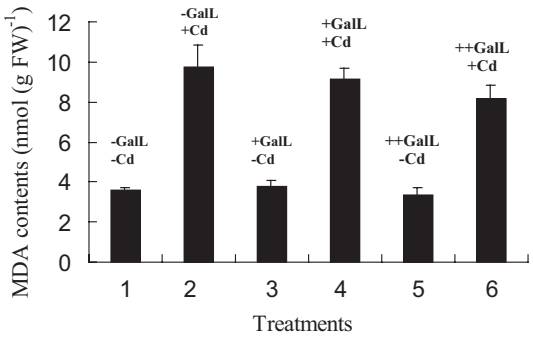

Figure 6: MDA contents in shoots of winter wheat seedlings grown in nutrient solution with different GalL (0, 25 mM) and Cd (0, 10, 25, 50 μM) treatments. Values are means ± S.D. of four replications.

3.2.3 Effects of GalL on POD activity

POD is an important antioxidant enzyme, catalyzing the decomposition of H_2O_2 to H_2O and O_2. The POD activities with GalL and Cd treatments are shown in Fig. 7. –GalL+Cd treatment had the lowest POD activity, and GalL addition significantly enhanced POD activity (p = 0.012). Under no Cd stress, GalL addition also marginally enhanced POD activity.

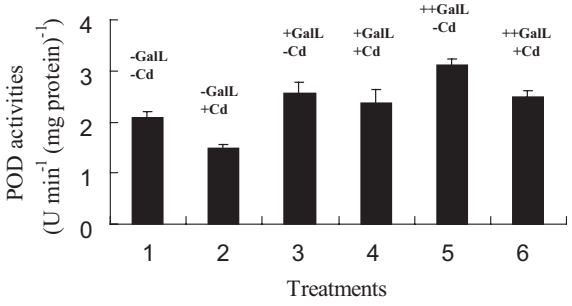

Figure 7: POD activities in shoots of winter wheat seedlings grown in nutrient solution with different GalL (0, 25 mM) and Cd (0, 10, 25, 50 μM) treatments. Values are means ± S.D. of four replications.

4 Discussion

Experiment 1 demonstrated that Cd significantly induced H_2O_2 and MDA accumulation in seedlings of winter wheat. In comparison to the control plants, H_2O_2 levels in plants exposed to 50 μM Cd were 6.5-fold higher in shoots and 20-fold higher in roots, and MDA levels were 3- and 4.5-fold higher (Figs. 2 and 4). Since *Van Assche* and *Clijsters* (1990) observed the induction of new peroxidase isoenzymes in roots and shoots of *Phaseolus vulgaris* upon Cd exposure, indicating for the first time that Cd exposure leads to the production of ROS, a great number of investigations have been reported for further details on Cd and oxidative stress (*Luo* et al., 1998; *Schützendübel* et al., 2001; *Olmos* et al., 2003; *Somashekaraiah* et al., 1992; *Chaoui* et al., 1997; *Shah* et al., 2001). Our finding is consistent with these previous reports.

According to the chemical properties of Cd and its biological significance, Cd belongs to the redox-inactive metals, with a lower redox potential than biological molecules. It cannot participate in biological redox reaction. Therefore, Cd cannot cause oxidative stress in plants directly.

A hypothetical model of Cd action on the cellular redox systems was proposed by *Schützendübel* et al. (2002). Cadmium, when absorbed by plants, readily induces biosynthesis of phytochelatins (PCs) which is associated with consumption of the antioxidant GSH. Depletion of GSH consequently causes a series of changes in the redox systems and accumulation of ROS such as H_2O_2. In plant cells, the most important reducing substrate for the detoxification of ROS is AsA (*Noctor* and *Foyer*, 1998). AsA is a powerful antioxidant that functions together with GSH and antioxidant enzymes to constitute the GSH–AsA cycle by which H_2O_2 is detoxified. Several reports have shown the role of AsA in alleviating ozone toxicity by detoxifying ROS (*Mächler* et al., 1995; *Moldau* et al., 1998; *Zheng* et al., 2000). Recently, *Maddison* et al. (2002) investigated the response of a sensitive radish genotype (*Raphanus sativus L.*) fed with L-Galactono-1, 4-lactone (GalL), the biosynthetic precursor of antioxidant ASA, to ozone toxicity. They showed that the sensitive radish genotype exhibited increased levels of AsA and an increased tolerance to ozone toxicity.

The essence of phytotoxicity of both ozone and heavy metals to plants is oxidative stress. According to the hypothesis of Cd action on the cellular redox systems and GSH–AsA cycle, increasing the level of the important antioxidant-AsA can alleviate the oxidative stress induced by Cd. In our experiment 2, addition of GalL decreased the accumulation of H_2O_2 (Fig. 5) and increased the activity of peroxidase (Fig. 7). The results indicated that addition of GalL in nutrient solution enhanced AsA biosynthesis in plants and thus increased the antioxidant capacity of plants. The findings provide evidence for the role of AsA in reducing Cd toxicity and, at the same time, support the hypothesis of Cd action on the cellular redox systems. Interestingly, we also found that although the levels of MDA, indicator of lipid peroxidation, increased significantly with Cd application as in experiment 1, they were not affected by GalL as significantly as H_2O_2. It is known that ROS accumulation readily results in lipid peroxidation and MDA contents usually correlate positively with H_2O_2 contents as we found in experiment 1. But in experiment 2, the data of MDA contents were not in agreement with H_2O_2 data and the above theory. There are possibly two reasons for this discrepancy: (1) MDA is an intermediate product not the end product of lipid peroxidation and thus, short-term treatment or determining MDA contents more often than once (not only 10 d after onset of Cd stress) would probably give a better picture of lipid peroxidation; (2) Cd possibly also directly induces lipid peroxidation, besides *via* the ROS way; this needs to be further studied.

Acknowledgments

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