

Impact of cadmium and hydrogen peroxide on ascorbate-glutathione recycling enzymes in barley root

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ABSTRACT

We analyse the effect of Cd and H₂O₂ short-term treatments on the activity of ascorbate-glutathione recycling enzymes in barley root tip. Even a short transient exposure of barley roots to low 15 µmol Cd concentration caused a marked approximately 70% root growth inhibition. Higher Cd concentrations caused root growth cessation during the first 6 h after short-term Cd treatment. Similarly, a marked root growth inhibition was also detected after the short-term exposure of barley seedlings to H₂O₂. Our results indicate that root ascorbate pool is more sensitive to Cd treatment than glutathione pool. Rapid activation of dehydroascorbate reductase and monodehydroascorbate reductase is the important component of stress response to the Cd-induced alterations in barley root tips. H₂O₂ is probably involved in the Cd-induced activation of monodehydroascorbate reductase, but it is not involved in the Cd-induced increase of dehydroascorbate reductase activity.

Keywords: dehydroascorbate reductase; glutathione reductase; monodehydroascorbate reductase; root growth inhibition

The increasing cadmium (Cd) contamination of soils due to various industrial and agricultural processes is a worldwide environmental problem. Due to the high solubility of cadmium in water it is easily taken up by plant roots leading to marked reduction in crop production. In addition, in several crop plants including barley, Cd is transported and accumulated in the upper part of plants including grains causing contamination of food chain ending with humans (Chen et al. 2007).

In plants, the earliest symptom of Cd toxicity is the inhibition of root growth as a consequence of disruption of several physiological processes including alterations in cell wall and plasma membrane, inhibition of various protein functions, modification of several signal transduction pathways, imbalance in water status, alterations in nutrient homeostasis and inhibition of cell mitosis and elongation (Benavides et al. 2005). However the most general Cd toxicity symptom is an increased oxidative damage arising from imbalance between

the generation and removal of reactive oxygen species – ROS (Sharma and Dietz 2009). Apart from involvement of ROS in phytotoxicity, they play a crucial role just in the activation of antioxidant systems and in the adaptation processes to altered environmental conditions (Dat et al. 2000). Recent studies provided evidence that hydrogen peroxide (H₂O₂) as a signal molecule is involved in the regulation of gene expression and enzymes activation during stress responses (Neill et al. 2002).

Ascorbate (ASC) is the most abundant antioxidant in plant cells and together with glutathione (GSH) contributes to the regulation of redox homeostasis in cells. Therefore, the rapid recycling of reduced ASC and GSH pool in plant tissues may contribute to enhanced tolerance to various stress conditions. It is confirmed by several transformed plants over-expressing ASC recycling enzymes, while their suppression in seedlings leads to increased sensitivity to stresses (Chen and Gallie 2005, Eltayeb et al. 2006, Yoshida et al. 2006, Li et

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al. 2010). Besides numerous other functions, GSH is involved also in ASC recycling as a reductant in dehydroascorbate reductase (DHAR) catalyzed reaction, therefore the maintenance of its reduced form by glutathione reductase (GR) is a crucial step in this system (Noctor et al. 2002).

It was previously reported that the prolonged Cd treatment increases ascorbate-glutathione metabolism and the activity of enzymes involved in their recycling in roots and leaves of wheat seedlings (Paradiso et al. 2008). The aim of this study was to analyse the effect of Cd and H₂O₂ short-term treatments on the activity of ascorbate-glutathione recycling enzymes – monodehydroascorbate reductase (MDHAR), DHAR and GR in barley root tip.

MATERIAL AND METHODS

Plant material and growth conditions. Barley seeds (*Hordeum vulgare* L.) cv. Slaven (Plant Breeding Station – Hordeum Ltd Sládkovičovo-Nový Dvůr, Slovak Republic) were imbibed in distilled water for 15 min followed by germination between two sheets of filter paper (density 110 g/m², Papírna Perštein, Czech Republic) moistened with distilled water in Petri dishes (5 mL per sheet of filter paper of 18 cm in diameter) at 25°C in darkness. The uniformly germinating seeds, 24 h after the onset of seed imbibition, were arranged into row between two sheets of filter paper moistened with distilled water in rectangle trays. Trays were placed into nearly vertical position to enable downward radical growth. Continuous moisture of filter papers was supplied from the reservoir with distilled water through the filter paper wick. Seedlings, with approximately 4 cm long roots, 60 h after the onset of seed imbibition were used for treatments. Roots of seedlings were immersed into distilled water (control); 10, 30, or 60 µmol CdCl₂; 0.1, 1 or 10 mmol H₂O₂ for 30 min. After washing in distilled water for 5 min the seedlings were incubated between two sheets of filter paper moistened with distilled water as described above. After 1, 2, 3, 4, 5 or 6 h of incubation after short-term treatments the individual barley root segments (3 mm in length) were obtained by the gradual cutting of each root from the tip to the base. The experiments were carried out in five independent series. The significance of differences between control and Cd- or H₂O₂-treated roots was analyzed using the Student's *t*-test.

Root length measurement. For the determination of root length increment, the positions of root tips following the treatments were marked on the filter paper. After 6 h roots were excised at the position of marks and the length increment was measured after recording with stereomicroscope (STMPRO BELPhotonics, Milano, Italy) using BEL micro image analyzer.

Protein extraction and sample preparation. The root segments were homogenized in a pre-cooled mortar with 100 mmol potassium phosphate extraction buffer pH 7.8 containing 1 mmol EDTA. After centrifugation at 12 000 × g for 10 min, proteins were quantified with bovine serum albumin as the calibration standard by the method of Bradford (1976).

Enzyme assays. Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was determined by measuring the decrease in absorbance at 340 nm reflecting the rate of NADH oxidation (Arrigoni et al. 1981). The reaction mixture contained 100 mmol potassium phosphate buffer pH 7.0, 1 mmol ascorbate, 0.2 mmol NADH, and 2 U of ascorbate oxidase from *Cucurbita* sp. (Sigma) and 10 µg of proteins from root extract. The reaction mixture was incubated at 30°C for 15 min.

Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was assayed by measuring the rate of ascorbate formation at 265 nm in reaction mixture containing 100 mmol potassium phosphate buffer pH 7.0, 1 mmol reduced glutathione, 0.5 mmol dehydroascorbate and 10 µg of proteins from root extract. The reaction mixture was incubated at 30°C for 15 min (Arrigoni et al. 1981).

Glutathione reductase (GR; EC 1.6.4.2) activity was determined by measuring the increase in absorbance at 412 nm reflecting the reduction of 5,5'-dithiodibis(2-nitrobenzoic acid) (DTNB) by reduced glutathione (Smith et al. 1988). The reaction mixture contained 50 mmol potassium phosphate buffer pH 7.8, 0.5 mmol EDTA, 0.5 mmol oxidized glutathione, 1 mmol DTNB, 0.25 mmol NADPH and 10 µg of proteins from root extract. The reaction mixture was incubated at 30°C for 30 min.

RESULTS AND DISCUSSION

The inhibition of root elongation is a very sensitive and common response of plants to several stress conditions including Cd. We demonstrated that even a short transient exposure of barley roots to low 15 µmol Cd concentration caused a

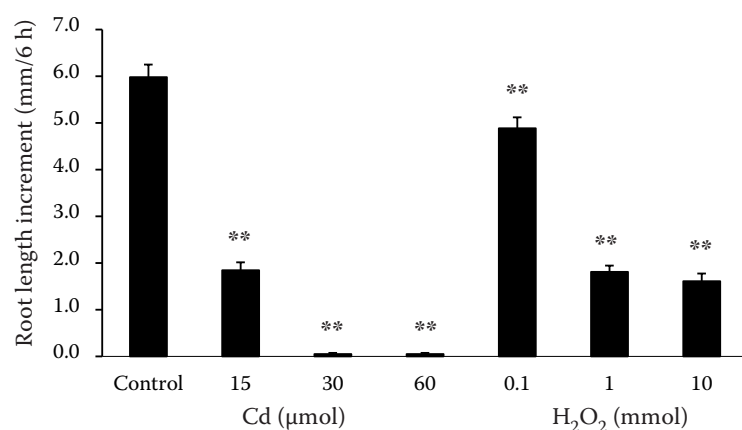


Figure 1. Root length increments 6 h after the short-term treatment with 0, 15, 30, 60 μmol Cd, or 0.1, 1, 10 mmol H_2O_2 . Mean values \pm SD ($n = 5$). * $P \leq 0.05$; ** $P \leq 0.01$

marked approximately 70% root growth inhibition (Figure 1). Higher 30 or 60 μmol Cd concentrations caused root growth cessation during the first 6 h after short-term Cd treatment. Similarly to what was observed for Cd treatments, a marked root growth inhibition was also detected after the short-term exposure of barley seedlings to H_2O_2 (Figure 1). However, despite the high mmol H_2O_2 concentration, root growth was not com-

pletely inhibited suggesting that apart from the Cd-induced H_2O_2 generation other processes were also involved in the Cd-induced root growth cessation (Introduction). On the other hand, due to the high amount of peroxidases in root tissues, a rapid decomposition of exogenously applied H_2O_2 may occur (Huttová et al. 2006).

Short-term treatment of barley roots with Cd or H_2O_2 caused a transient decrease of MDHAR activ-

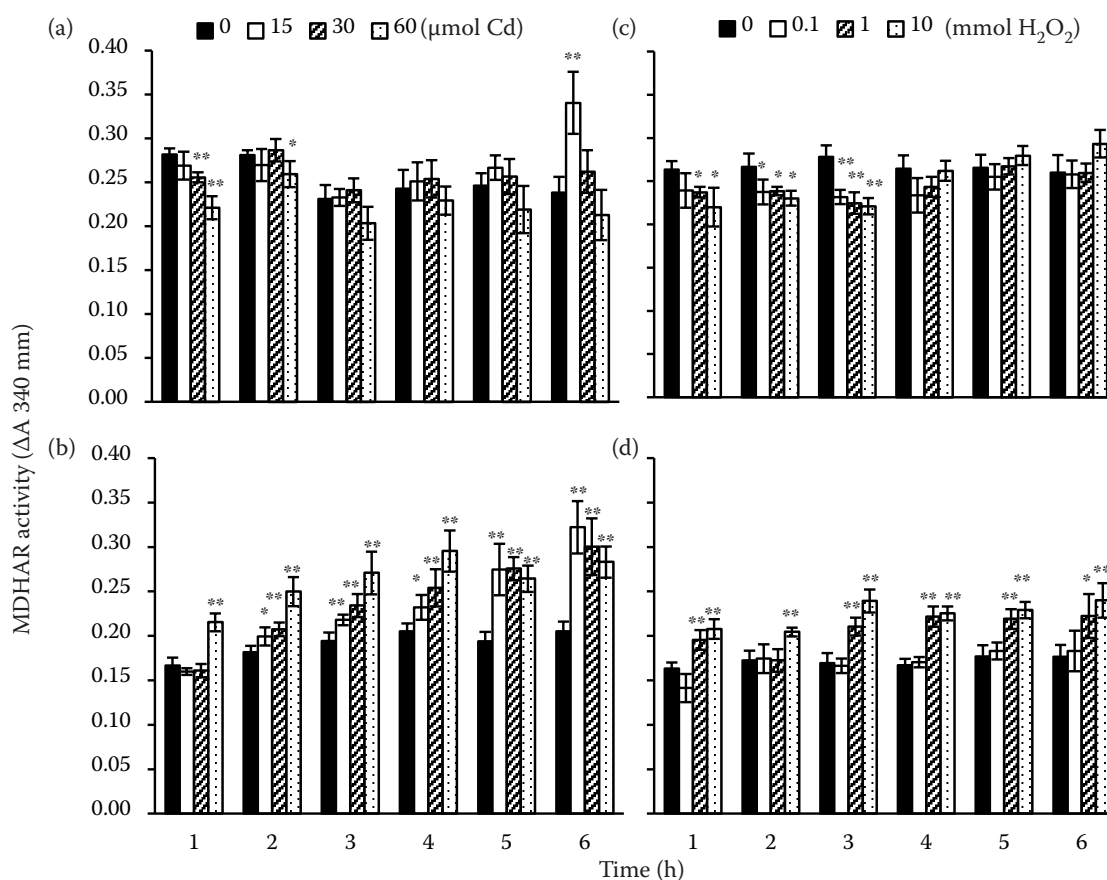


Figure 2. Monodehydroascorbate reductase (MDHAR) activity 1, 2, 3, 4, 5 and 6 h after short-term treatment (30 min) with 0 (control), 15, 30 or 60 μmol Cd (a, b) and 0.1, 1, or 10 mmol H_2O_2 (c, d) in the first (a, c) and second (b, d) 3 mm long root segments behind the root apex. Mean values \pm SD ($n = 5$). * $P \leq 0.05$; ** $P \leq 0.01$

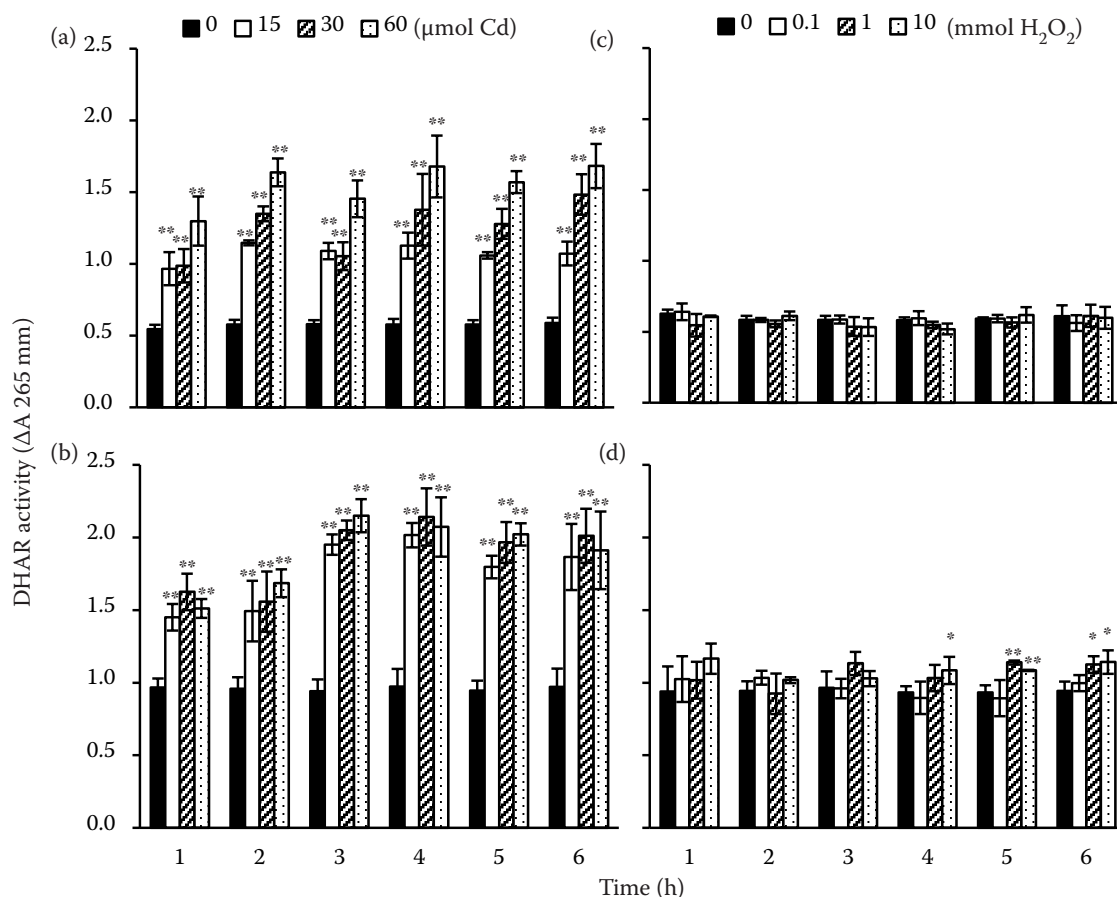


Figure 3. Dehydroascorbate reductase (DHAR) activity 1, 2, 3, 4, 5 and 6 h after short-term treatment (30 min) with 0 (control), 15, 30 or 60 $\mu\text{mol Cd}$ (a, b) and 0.1, 1, or 10 $\text{mmol H}_2\text{O}_2$ (c, d) in the first (a, c) and second (b, d) 3 mm long root segments behind the root apex. Mean values \pm SD ($n = 5$). * $P \leq 0.05$; ** $P \leq 0.01$

ity in the first root segment immediately behind the root apex containing meristematic and elongation zone (Figures 2a,c). In contrast to this segment, in the second root segment containing the beginning of differentiation zone the activity of MDHAR raised both after Cd or H₂O₂ short-term treatments in a concentration dependent manner (Figures 2b,d). However this activation was more relevant after the Cd treatment than after the H₂O₂ exposure. It was previously shown that the level of ASC and its redox state is markedly increased in MDHAR transgenic tobacco plants compared to control, which contributes to lower H₂O₂ level and enhanced tolerance to ozone, salt and osmotic stresses (Eltayeb et al. 2007). In addition, apart from the enhanced stress tolerance, MDHAR expressing transgenic rice plants showed better yield attributes compared to non-transgenic lines (Sultana et al. 2012).

The short-term treatment of roots by Cd resulted in the greatest changes in DHAR activity (Figures 3a,b). It was activated within 1 h of Cd treatment along the whole barley root tip. However, in the first seg-

ment (0–3 mm behind the root apex) DHAR activity increased in a Cd concentration dependent manner, in the second root segment even a low concentration of Cd (15 μmol) caused a marked increase of DHAR activity similarly to higher Cd concentrations. In contrast to Cd treatment, the exposure of root to H₂O₂ did not stimulate the activity of DHAR, only a slight increase was observed 5 h and 6 h after the short-term treatment with higher H₂O₂ concentrations in the second root segment (Figures 3c,d). These results suggest that Cd does not affect the redox state of ASC only through the action of Cd-induced accumulation of H₂O₂. In addition, in our previous work we showed that Cd has an opposite effect on the expression of genes encoding different isoforms of ascorbate peroxidase, but the total activity of ascorbate peroxidase was not affected (Bočová et al. 2012a). ASC plays a crucial role mainly in the apoplast, where it is the sole low molecular weight antioxidant. In addition, it is a substrate for ascorbic acid oxidase generating monodehydroascorbate as a natural electron acceptor of plasma

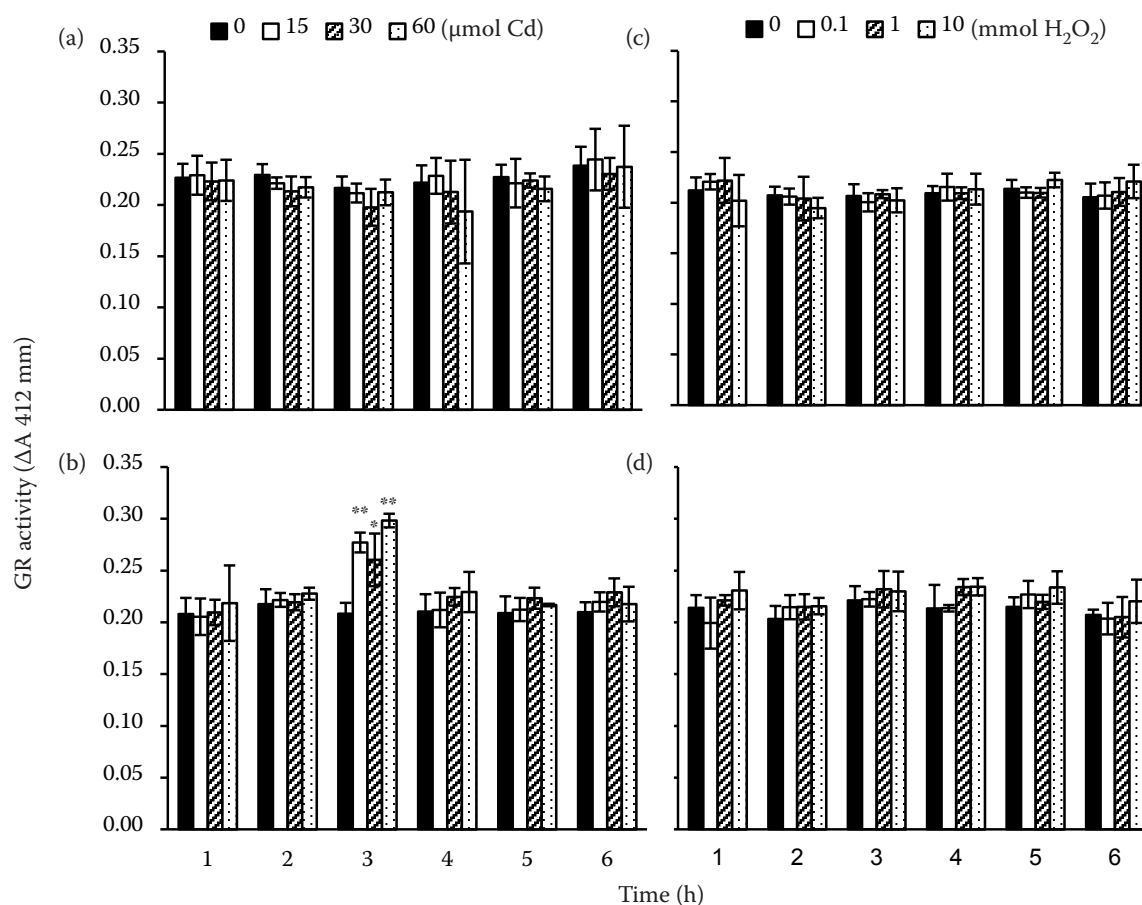


Figure 4. Glutathione reductase (GR) activity 1, 2, 3, 4, 5 and 6 h after short-term treatment (30 min) with 0 (control), 15, 30 or 60 $\mu\text{mol Cd}$ (a, b) and 0.1, 1, or 10 mmol H_2O_2 (c, d) in the first (a, c) and second (b, d) 3 mm long root segments behind the root apex. Mean values \pm SD ($n = 5$). * $P \leq 0.05$; ** $P \leq 0.01$

membrane electron transport system inevitable for nutrient uptake and cell growth (Smirnov 1996). In our previous work we showed that Cd rapidly disturbs apoplastic ASC redox status (Bočová et al. 2012b), which may be involved in the activation of ascorbate recycling enzymes. The cytosolic form of DHAR is well documented, but some results showed its localization also in the apoplastic spaces of plant tissues (Vanacker et al. 1998). Transgenic tobacco plants overexpressing *Arabidopsis* DHAR showed better root growth than wild type plants under Al stress due to the lower H_2O_2 content and lipid peroxidation as a consequence of higher reduced ASC in root and ascorbate peroxidase activity (Yin et al. 2010).

In contrast to the activity of MDHAR or DHAR, the activity of GR was only transiently increased 3 h after the short-term Cd treatment in the second root segment (3–6 mm behind the root apex) containing the beginning of differentiation zone, while H_2O_2 did not affect its activity (Figure 4). Probably, the short-term treatments were insufficient to induce

GSH depletion and stimulation of GR activity. In wheat, prolonged Cd treatment markedly increased GR activity in root tissues (Yannarelli et al. 2007). Similarly to this and our observation Wu et al. (2004) reported that the level of ASC decreased even at low Cd concentrations, while the GSH level raised at low and decreased only after the prolonged exposure of seedlings to high Cd concentration.

In conclusion, our results indicate that root ASC pool is more sensitive to Cd treatment than GSH pool. Rapid activation of DHAR and MDHAR is the important component of stress response to the Cd-induced alterations in barley root tips. H_2O_2 is probably involved in the Cd-induced activation of MDHAR, but it is not involved in the Cd-induced increase of DHAR activity.

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