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

**V. Zelinová, B. Bočová, J. Huttová, I. Mistrík, L. Tamás**

*Institute of Botany, Slovak Academy of Sciences, Bratislava, Slovak Republic*

**ABSTRACT**

We analyse the effect of Cd and $H_2O_2$ 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 $H_2O_2$. 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. $H_2O_2$ 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 ($H_2O_2$) 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

Supported by the Slovak Grant Agency of the Ministry of Education and Slovak Academy of Sciences – VEGA, Grant No. 2/0019/13.

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 $H_2O_2$ 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 Dvor, Slovak Republic) were imbibed in distilled water for 15 min followed by germination between two sheets of filter paper (density 110 g/m$^2$, Papírna Perštejn, 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 $CdCl_2$; 0.1, 1 or 10 mmol $H_2O_2$ 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 $H_2O_2$-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

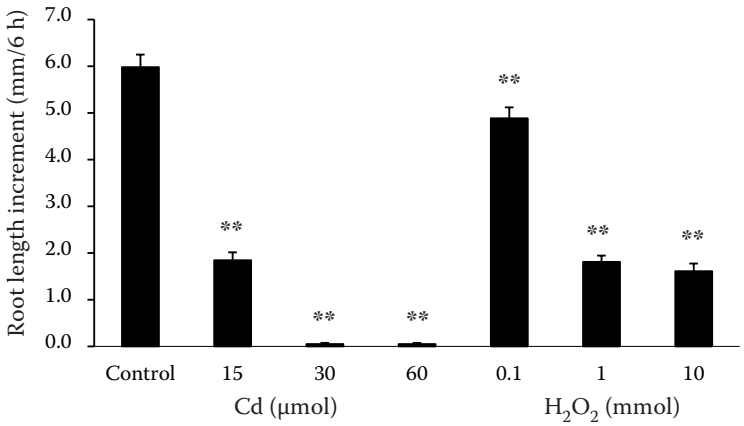


Figure 1. Root length increments 6 h after the short-term treatment with 0, 15, 30, 60 μmol Cd, or 0.1, 1, 10 mmol $H_2O_2$. Mean values ± SD ($n$ = 5). *$P \leq 0.05$; **$P \leq 0.01$

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 completely 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-

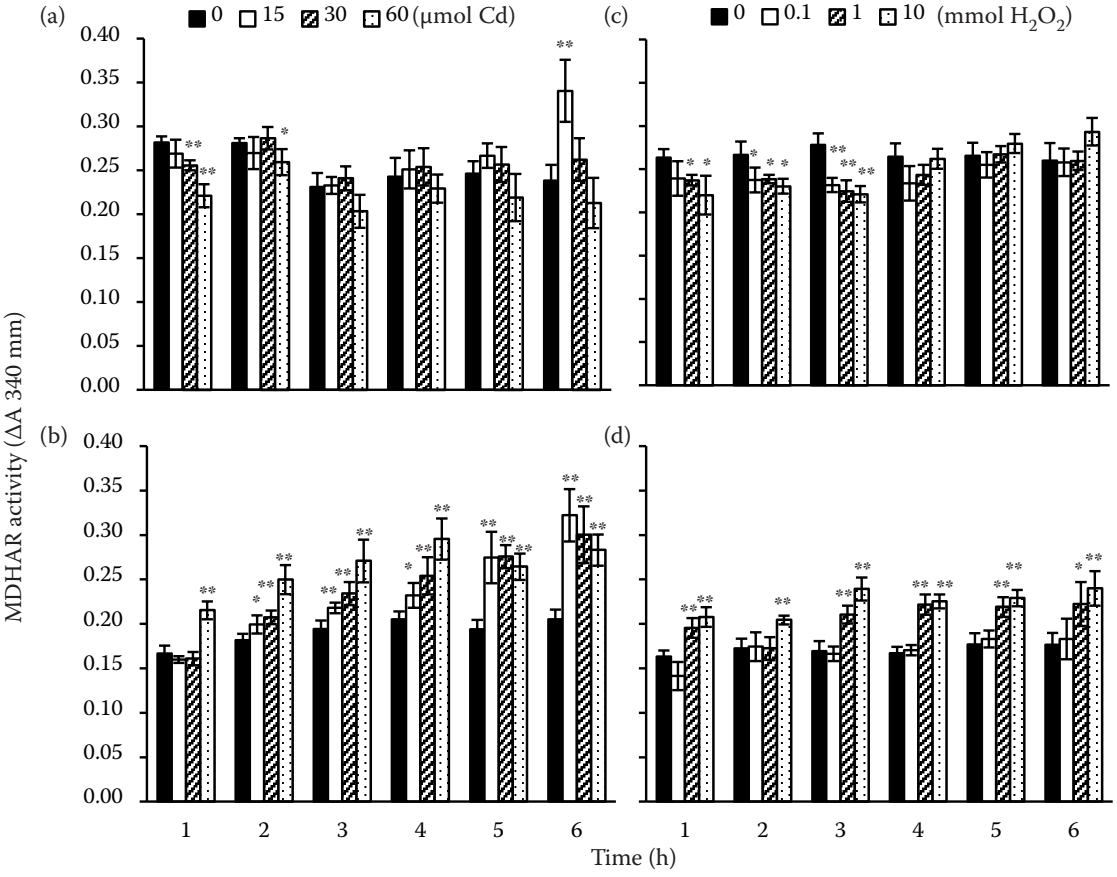


Figure 2. Monodehydroascorbate reductase (MDHAR) activity 1, 2, 3, 4, 5 and 6 h after short-term treatment (30 min) with 0 (control), 15, 30 or 60 μmol Cd (a, b) and 0.1, 1, or 10 mmol $H_2O_2$ (c, d) in the first (a, c) and second (b, d) 3 mm long root segments behind the root apex. Mean values ± SD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

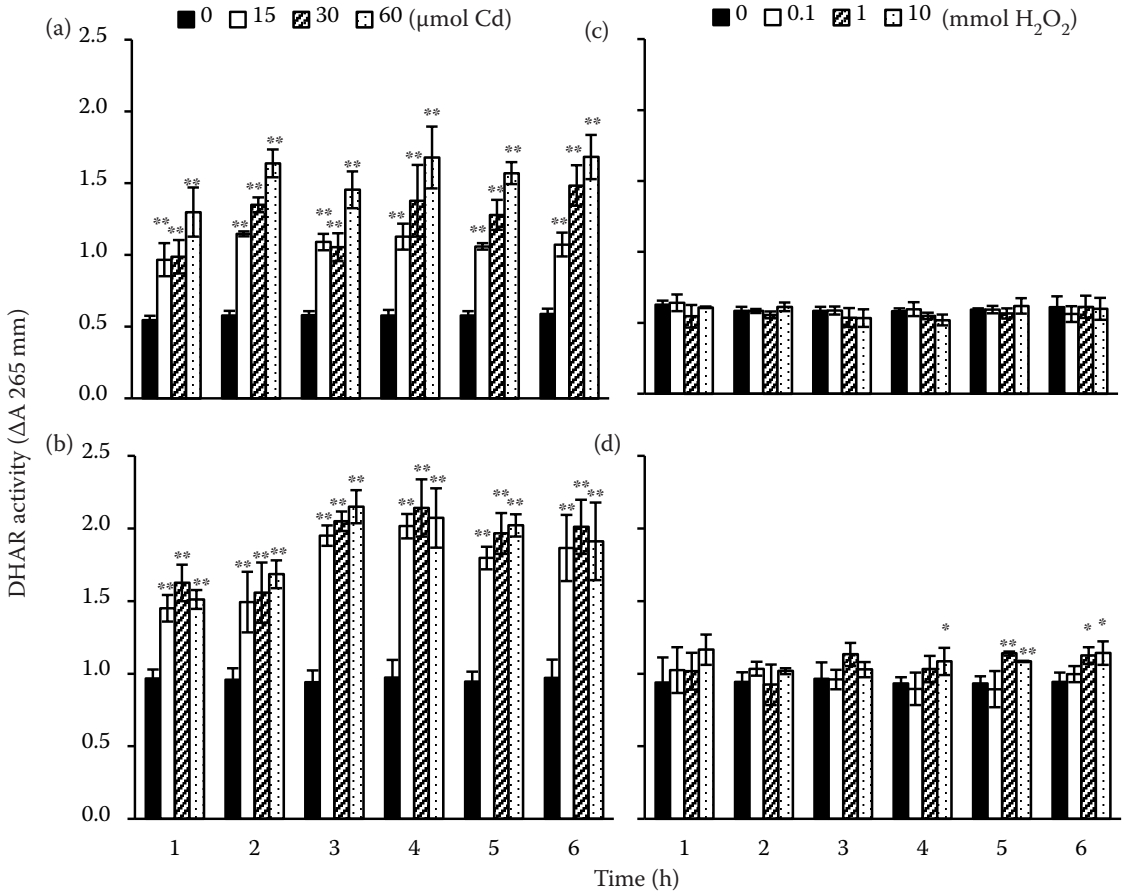


Figure 3. Dehydroascorbate reductase (DHAR) activity 1, 2, 3, 4, 5 and 6 h after short-term treatment (30 min) with 0 (control), 15, 30 or 60 µmol Cd (a, b) and 0.1, 1, or 10 mmol $H_2O_2$ (c, d) in the first (a, c) and second (b, d) 3 mm long root segments behind the root apex. Mean values ± SD ($n$ = 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 $H_2O_2$ short-term treatments in a concentration dependent manner (Figures 2b,d). However this activation was more relevant after the Cd treatment than after the $H_2O_2$ 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 $H_2O_2$ 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 segment (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 $H_2O_2$ 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 $H_2O_2$ 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 $H_2O_2$. 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

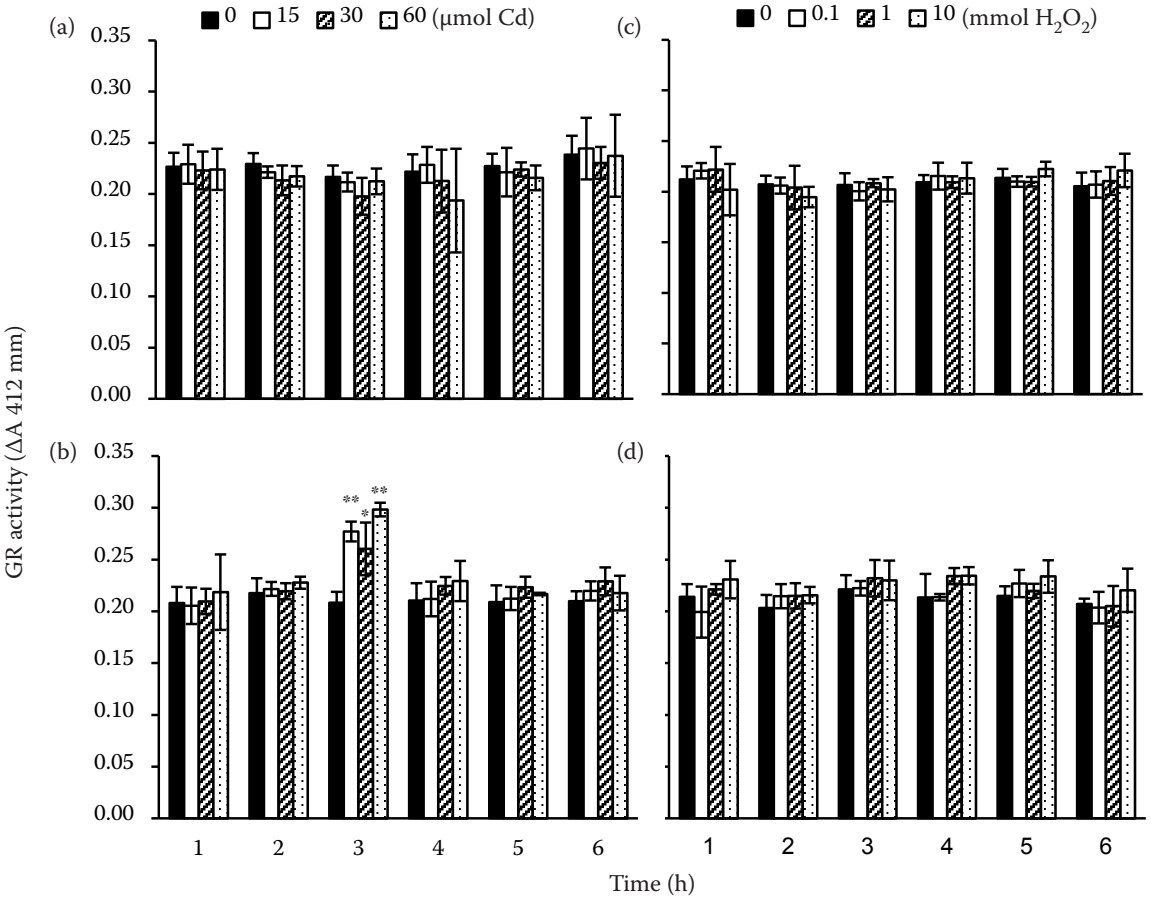


Figure 4. Glutathione reductase (GR) activity 1, 2, 3, 4, 5 and 6 h after short-term treatment (30 min) with 0 (control), 15, 30 or 60 μmol Cd (a, b) and 0.1, 1, or 10 mmol $H_2O_2$ (c, d) in the first (a, c) and second (b, d) 3 mm long root segments behind the root apex. Mean values ± SD ($n$ = 5).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

membrane electron transport system inevitable for nutrient uptake and cell growth (Smirnoff 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 lipide 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.

**Acknowledgements**

We wish to thank Margita Vašková for excellent technical assistance.

## REFERENCES


Arrigoni O., Dipierro S., Borraccino G. (1981): Ascorbate free radical reductase, a key enzyme of the ascorbic acid system. FEBS Letter, *125*: 242–245.

Benavides M.P., Gallego S.M., Tomaro M.L. (2005): Cadmium toxicity in plants. Brazilian Journal of Plant Physiology, *17*: 21–34.

Bočová B., Huttová J., Liptáková Ľ., Mistrík I., Ollé M., Tamás L. (2012a): Impact of short-term cadmium treatment on catalase and ascorbate peroxidase activities in barley root tips. Biologia Plantarum, *56*: 724–728.

Bočová B., Mistrík I., Pavlovkin J., Tamás L. (2012b): Cadmium disrupts apoplastic ascorbate redox status in barley root tips. Acta Physyologiae Plantarum. DOI 10.1007/s11738-012-1030-y (In press)

Bradford M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, *72*: 248–254.

Chen Z., Gallie D.R. (2005): Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. Plant Physiology, *138*: 1673–1689.

Chen F., Wu F., Dong J., Vincze E., Zhang G., Wang F., Huang Y., Wei K. (2007): Cadmium translocation and accumulation in developing barley grains. Planta, *227*: 223–232.

Dat J., Vandenabeele S., Vranová E., Van Montagu M., Inzé D., Van Breusegem F. (2000): Dual action of the active oxygen species during plant stress responses. Cellular and Molecular Life Sciences, *57*: 779–795.

Eltayeb A.E., Kawano N., Badawi G.H., Kaminaka H., Sanekata T., Morishima I., Shibahara T., Inanaga S., Tanaka K. (2006): Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol. Physiologia Plantarum, *127*: 57–65.

Eltayeb A.E., Kawano N., Badawi G.H., Kaminaka H., Sanekata T., Shibahara T., Inanaga S., Tanaka K. (2007): Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. Planta, *225*: 1255–1264.

Huttová J., Mistrík I., Ollé-Šimonovičová M., Tamás L. (2006): Cadmium induced changes in cell wall peroxidase isozyme pattern in barley root tip. Plant, Soil and Environment, *52*: 250–253.

Li F., Wu Q.Y., Sun Y.L., Wang L.Y., Yang X.H., Meng Q.W. (2010): Overexpression of chloroplastic monodehydroascorbate reductase enhanced tolerance to temperature and methyl viologen-mediated oxidative stress. Physiologia Plantarum, *139*: 421–434.

Neill S., Desikan R., Hancock J. (2002): Hydrogen peroxide signalling. Current Opinion in Plant Biology, *5*: 388–395.

Noctor G., Gomez L., Vanacker H., Foyer C.H. (2002): Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. Journal of Experimental Botany, *53*: 1283–1304.

Paradiso A., Berardino R., de Pinto M.C., Sanitá di Toppi L., Storelli M.M., Tommasi F., De Gara L. (2008): Increase in ascorbate-glutathione metabolism as local and precocious systemic responses induced by cadmium in durum wheat plants. Plant and Cell Physiology, *49*: 362–374.

Sharma S.S., Dietz K.J. (2009): The relationship between metal toxicity and cellular redox imbalance. Trends in Plant Science, *14*: 43–50.

Smirnoff N. (1996): The function and metabolism of ascorbic acid in plants. Annals of Botany, *78*: 661–669.

Smith I.K., Vierheller T.L., Thorne C.A. (1988): Assay of glutathione reductase in crude tissue homogenates using 5,5`-dithiobis(2-nitrobenzoic acid). Analytical Biochemistry, *175*: 408–413.

Sultana S., Khew C.Y., Morshed M.M., Namasivayam P., Napis S., Ho C.L. (2012): Overexpression of monodehydroascorbate reductase from a mangrove plant (AeMDHAR) confers salt tolerance on rice. Journal of Plant Physiology, *169*: 311–318.

Vanacker H., Carver T.L.W., Foyer C.H. (1998): Pathogen-induced changes in the antioxidant status of the apoplast in barley leaves. Plant Physiology, *117*: 1103–1114.

Wu F.B., Chen F., Wei K., Zhang G.P. (2004): Effect of cadmium on free amino acids, glutathione and ascorbic acid concentrations in two barley genotypes (*Hordeum vulgare* L.) differing in cadmium tolerance. Chemosphere, *57*: 447–454.

Yannarelli G.G., Fernández-Alvarez A.J., Santa-Cruz D.M., Tomaro M.L. (2007): Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress. Phytochemistry, *68*: 505–512.

Yin L., Wang S., Eltayeb A.E., Uddin M.I., Yamamoto Y., Tsuji W., Takeuchi Y., Tanaka K. (2010): Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. Planta, *231*: 609–621.

Yoshida S., Tamaoki M., Shikano T., Nakajima N., Ogawa D., Ioki M., Aono M., Kubo A., Kamada H., Inoue Y., Saji H. (2006): Cytosolic dehydroascorbate reductase is important for ozone tolerance in *Arabidopsis thaliana*. Plant and Cell Physiology, *47*: 304–308.


Received on September 1, 2012
Accepted on December 18, 2012

*Corresponding author:*

Dr. Ladislav Tamás, Slovak Academy of Sciences, Institute of Botany, Dúbravská cesta 9, 845 23 Bratislava, Slovak Republic
phone: + 421 2 5942 6116, fax: + 421 2 5477 1948, e-mail: Ladislav.Tamas@savba.sk