BIOLOGIA PLANTARUM 47 (1): 71-78, 2003/4

The activity of the antioxidative system in cadmium-treated *Arabidopsis thaliana*

E. SKÓRZYŃSKA-POLIT*, M. DRĄŻKIEWICZ and Z. KRUPA

Department of Plant Physiology, Maria Curie-Skłodowska University, Akademicka 19, PL-20033 Lublin, Poland

Abstract

Changes in the content of reactive oxygen species (ROS) and activity of the antioxidant system were measured in leaves of *Arabidopsis thaliana* (L.) Heynh exposed to Cd^{2+}. Mature plants growing in the nutrient solution were treated with Cd^{2+} at different concentrations (0, 5, 25, 50, 100 μM). An increase of $O_2^{\cdot-}$ content in leaves was observed at 5, 25 and 50 μM Cd^{2+}. A strong accumulation of H_2O_2 was found only at the lowest Cd^{2+} concentration. The content of $OH^{\cdot}$ was high at 50 and 100 μM Cd^{2+}. Superoxide dismutase (SOD) activity was always higher in Cd^{2+}-treated plants than in control. Catalase (CAT) activity decreased with increasing Cd^{2+} concentration in the nutrient solution. Guaiacol peroxidase (POX) activity was particularly high at the lowest and highest Cd^{2+} concentrations and ascorbate peroxidase (APX) activity additionally at 50 μM Cd^{2+}. Enhanced activity of monodehydroascorbate reductase (MDHAR) and strong reduction in ascorbate (AA) content were observed at 25 μM Cd^{2+}. Glutathione reductase (GR) activity was always higher than in control but decreased as Cd^{2+} concentration increased. However, it was accompanied by gradual content increase of SH-groups.

Additional key words: ascorbate, catalase, glutathione, dehydroascorbate, peroxidases, reactive oxygen species, superoxide dismutase.

Introduction

Reactive oxygen species such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) are formed in metabolic processes occurring in plants (for details see Scandalios 1993, Vácha 1995, Bartosz 1997), but when they are created on non-controlled levels they can lead to disturbances and injury in cell metabolism or even to cell death (for details see Salin 1987). The antioxidative system consisting of several enzymes and antioxidants fulfils a protective role and stabilizes the amounts of reactive oxygen species (ROS) in plant cells (for details see Sies 1993). This system includes enzymes such as catalase (CAT), peroxidases (POXs), superoxide dismutase (SOD), and low molecular mass antioxidants like ascorbate (AA), glutathione (GSH), tocopherols, carotenoids (Cadenas 1989, Bartosz 1997). The glutathione-ascorbate cycle protects first of all the photosynthetic apparatus against toxic action of reactive oxygen species in chloroplasts of higher plants. It consists of enzymes such as: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and non-enzymatic factors (AA, GSH). These low molecular mass antioxidants participate in a cyclic transfer of reducing equivalents (Noctor and Foyer 1998). Changes in the antioxidative defence system were observed during water- (Boo and Jung 1999) ozone- (Rao *et al.* 1995, Pell *et al.* 1997, Niewiadomska *et al.* 1999), drought- (Smirnoff and Colombé 1988, Loggini *et al.* 1999) or xenobiotic-stresses (Knörzner *et al.* 1996). Changes in functioning of

Received 30 April 2002, *accepted* 15 October 2002.

Abbreviations: AA - ascorbate; APX - ascorbate peroxidase; CAT - catalase; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; DMSO - dimethyl sulfoxide; DTNB - 5,5'-dithiobis(2-nitrobenzoic acid); GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidised glutathione; MDHAR - monodehydroascorbate reductase; MSA - methane sulfinic acid; NBT - nitroblue tetrazolium; POX - guaiacol peroxidase; SOD - superoxide dismutase; TNB - 5-thio-2-nitrobenzoic acid.

Acknowledgements: This research was supported by State Committee for Scientific Research (KBN), grant No 6P04C 064 15.

* Fax (+48) 81 5375901, e-mail: eskorzyn@biotop.umcs.lublin.pl

the antioxidative system are also known upon heavy metals stress (Gallego *et al.* 1996, Weckx and Clijsters 1996, 1997, Mazhoudi *et al.* 1997, Schickler and Caspi 1999, Stroiński 1999). The formation of total free radicals and activities of antioxidant enzymes were measured only in lead-treated lupin roots (Rucińska *et al.* 1999). The action of heavy metals on the antioxidant system is still a very interesting problem. Although Cd^{2+} toxicity to plants is well documented, the mechanism of its action is still unclear (Barceló and Poschenrieder 1990, Woźny *et al.* 1990, Krupa and Baszyński 1995, Prasad 1996, Krupa 1999). The aim of the present studies was to examine whether some changes in content of the individual ROS and functioning of antioxidant system (enzyme activities and content of non-enzymatic antioxidants) occurred in Cd^{2+}-treated model plant *Arabidopsis thaliana.*

Materials and methods

Plants of *Arabidopsis thaliana* (L.) Heynh cv. Columbia were grown in the soil at day/night temperature of 23/19 °C and photosynthetic photon flux density of 140 μmol m^{-2} s^{-1}, under a 11-h photoperiod for 7 weeks, then they were transferred into modified Hoagland solution and after 4 d they were treated with different concentrations of Cd^{2+}: 0 (control), 5, 25, 50, 100 μM in the form $CdSO_4 . H_2O$. After 7 d of heavy metal treatment leaves were harvested. Cut leaves from at least 5 plants (per each of individual Cd^{2+} concentrations and control) were mixed, weighed, frozen in liquid nitrogen and analysed.

The plant material was ground to a fine powder in liquid nitrogen and homogenized in extraction buffer (50 mM phosphate buffer, pH 7.0) according to Milosevic and Slusarenko (1996), at the ratio: 1 g of frozen leaves per 2 cm^3 ice-cold extraction buffer when ROS content was estimated and 1 g the plant material per 4 cm^3 of ice-cold extraction buffer when enzymatic activities were determined. To estimate hydroxyl radical content 1 g of frozen in liquid nitrogen leaves was homogenized in 2 cm^3 of 5 % ice-cold dimethyl sulfoxide (DMSO). To determine the metabolites content 1 g of frozen plant material was ground in 5 cm^3 of 5 % ice-cold *m*-phosphoric acid. All procedures were performed at 4 °C. The supernatant was collected by centrifugation (15 000 *g* at 4 °C for 20 min) and immediately used to individual assays.

Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as standard.

The content of superoxide radicals was estimated according to Green and Hill (1984) measuring differences in absorbance (A_{490}) by *Shimadzu UV-160A* spectrophotometer (Kyoto, Japan) thermostated chamber *CPS 240A* (*Shimadzu*), due to superoxide-dependent reduction of nitroblue tetrazolinum (NBT) to formazan in the reaction mixture containing SOD and without it. The final volume was 1 cm^3.

The content of H_2O_2 was determined on a basis of horseradish peroxidase-dependent oxidation of phenol red leading to the formation of a compound that, at an alkaline pH, increase A_{600} (Pick 1986).

Hydroxyl radical was determined as methane sulfinic acid (MSA) according to Babbs *et al.* (1989). The leaves conserved in the nitrogen were ground in 5 % dimethyl sulfoxide (DMSO) to trap hydroxyl radical and centrifuged. The colour reaction was started by addition of 0.07 cm^3 of 30 mM Fast blue RR salt (*Sigma*, St. Louis, USA) to previously acidified plant extract. The probes were incubated in the dark at room temperature for 10 min. Then 1 cm^3 of toluene/*n*-butanol (3:1, v/v) was added and the samples were centrifuged at 500 *g* for 1.5 min. The absorbance of the samples was measured at A_{425} using a blank prepared with 1 cm^3 5 % DMSO.

The spectrophotometric assay of catalase activity (CAT) [EC 1.11.1.6] was performed after the method of Aebi (1984). Enzyme preparation (0.02 cm^3) was added to 1.5 cm^3 of freshly prepared 10 mM solution of H_2O_2 in 50 mM phosphate buffer (pH 7.0). The decrease in A_{240} was measured for 90 s at 25 °C.

To determine activity of guaiacol peroxidase (POX) [EC 1.11.1.7] the leaf extract (0.015 cm^3) was added to 1 cm^3 of 100 mM phosphate buffer pH 6.25 containing 0.012 % guaiacol and 0.03 % H_2O_2 (Milosevic and Slusarenko 1996). The increase in A_{470} was measured for 3 min.

Total activity of superoxide dismutase (SOD) [EC 1.15.1.1] was measured as the inhibition of NBT reduction provoked by superoxide generated *via* the xanthin/xanthin oxidase system at A_{560} (Beauchamp and Fridovich 1971). One unit of SOD activity was defined as the amount of enzyme necessary to inhibit the reduction of NBT by 50 %.

Ascorbate peroxidase activity (APX) [EC 1.11.1.11] was determined according to Nakano and Asada (1987), using a reaction mixture containing 50 mM phosphate buffer pH 7.0, 0.1 mM hydrogen peroxide, 0.5 mM ascorbate and enzyme extract (0.02 cm^3) in final 1 cm^3 volume. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in A_{290}.

Monodehydroascorbate reductase (MDHAR) [EC 1.1.5.4] and dehydroascorbate reductase (DHAR) [EC 1.8.5.1] activities were assayed according to Miyake and Asada (1992). MDHAR was assayed by following decrease in A_{340} due to the oxidation of NADPH. The reaction mixture contained 50 mM HEPES-NaOH, pH 7.6, 0.1 mM NADPH, 2.5 mM ascorbate, 0.01 cm^3 of

ascorbate oxidase and 0.1 cm^3 of extract. DHAR activity was measured as an increase in A_{265} for 30 s. The reaction mixture contained 50 mM phosphate buffer pH 7.0, 2.5 mM GSH, 0.2 mM DHA, 0.1mM EDTA and 0.1 cm^3 of extract in a final volume of 1 cm^3.

The activity of glutathione reductase (GR) [EC 1.6.4.2] was measured according to Milosevic and Slusarenko (1996) as an increase in A_{412} caused by the reduction of DTNB to TNB by reduced glutathione produced in the reaction. Enzyme extract was added to 1 cm^3 of 100 mM phosphate buffer (pH 7.5) containing 1 mM DTNB and 1 mM NADPH. The reaction was started by adding 0.01cm^3 of 10 mM GSSG and proceeded at 30 °C in thermostated chamber *CPS 240A* of spectrophotometer *Shimadzu UV-160A* for 5 min.

Ascorbate (AA) and dehydroascorbate (DHA) content were determined as described Wang *et al.* (1991). This assay was based on the reduction of ferric ion to ferrous ion with ascorbic acid in acid solution followed by formation of the red chelate between ferrous ion and bathophenanthroline that absorbs at 534 nm. The total ascorbate (AA + DHA) was determined through DHA reduction to AA by dithiotreitol. DHA concentration was calculated from the difference of "total ascorbate" and AA concentration.

GSH and GSSG content were measured by the method of Anderson (1985) using 2-vinylpyridine to reaction with SH-groups. The content of SH-groups was calculated from the difference of the total SH-groups and GSSG.

The estimated values are the means of samples from three independent experiments each at least in 5 replicates ± SEM.

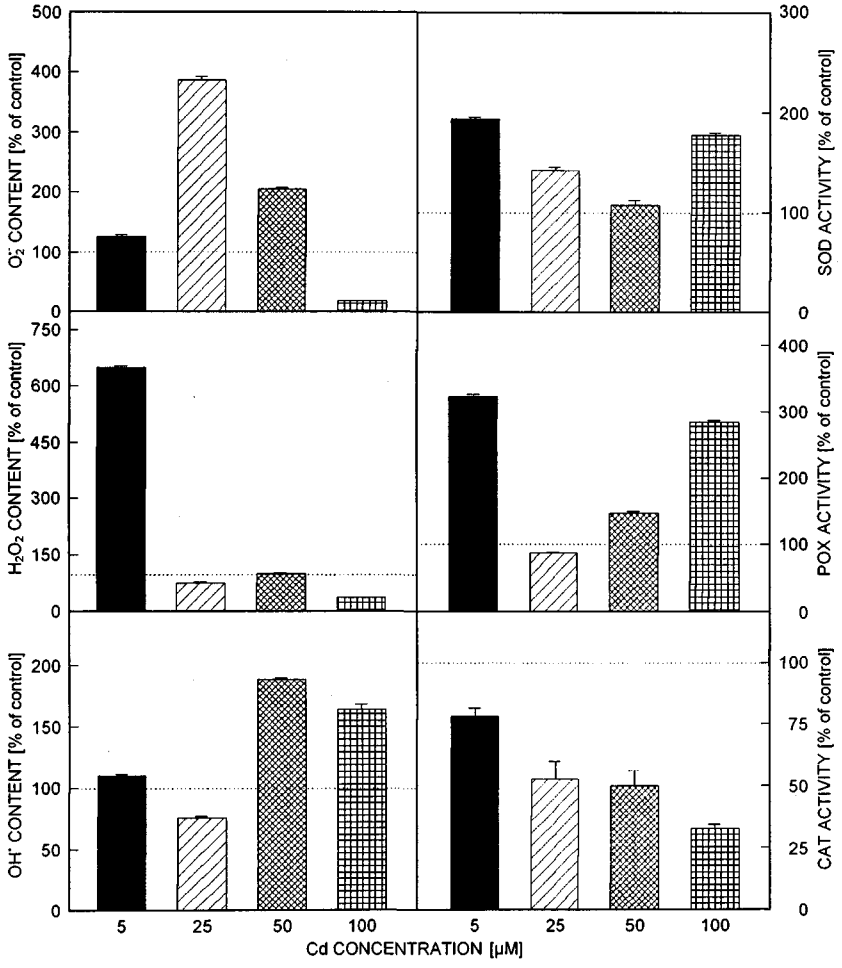

Fig. 1. The ROS content and activity of the antioxidant enzymes in leaves of *Arabidopsis thaliana* growing for 7 d in the nutrient solution with different Cd concentration. In leaves of control plants the level of $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$ was 5.28 ± 0.21 nmol(NBT_{red}) g^{-1}(f.m), 4.76 ± 0.06 μmol g^{-1}(f.m.) and 0.95 ± 0.31μmol(MSA) g^{-1}(f.m.), respectively. In control plants activity of SOD was 19.60 ± 0.22 units mg^{-1}(protein), activity of POX expressed as ΔA_{470} mg^{-1}(protein) min^{-1} was 5.85 ± 0.07; activity of CAT expressed as ΔA_{240} mg^{-1}(protein) min^{-1} was 1.845 ± 0.10.

Results

At 5, 25 and 50 μM Cd^{2+} in the nutrient solution an increase in $O_2^{\cdot-}$ content in leaves of *Arabidopsis thaliana* plants was observed (to 126, 387, 200 % of control, respectively). However at 100 μM Cd^{2+} $O_2^{\cdot-}$ content decreased to 19 % in comparison with control (Fig. 1).

Strong accumulation of H_2O_2 (to 650 % of control) was measured only at 5 μM Cd^{2+}. At higher metal concentrations the content of this ROS was lower than in control. At 50 and 100 μM Cd^{2+} $OH^{\cdot}$ content was higher than control (190 % and 165 % of control, respectively).

The activity of SOD was always higher in Cd^{2+}-treated plants than in control (Fig. 1). At 5 μM Cd^{2+} SOD activity reached 194 % of control (Fig. 1) then it gradually decreased with increasing Cd^{2+} concentration but at 100 μM Cd^{2+} its activity again increased to 178 % of control. POX activities were particularly high at 5 and 100 μM Cd^{2+} (337 % and 299 % of control, respectively). CAT activity was always lower than control and it dropped to 33 % of control at 100 μM Cd^{2+} (Fig. 1).

Elevated activity of APX was measured at 5 μM Cd^{2+}, but at 50 and 100 μM Cd^{2+} it was considerably higher (approx. 2 times) than in control (Fig. 2). Activity of MDHAR was higher in comparison with control only at 25 μM Cd^{2+}. DHAR activity was enhanced at 5 μM Cd^{2+} but at higher Cd^{2+} concentrations in the nutrient solution it was lower than control. GR activity was always higher than control but it decreased gradually with increasing metal concentration.

The AA + DHA content was approx. 2 times higher than control only at the lowest Cd^{2+} concentration (Fig. 3). It was caused by high DHA accumulation. AA content was elevated at 5 and 50 μM Cd^{2+}. Strong reduction in AA content to 50 % of control was observed at 25 μM Cd^{2+}.

An increase of SH-group + GSSG content was observed in Cd^{2+}-treated plants (Fig. 3). The content of SH-groups as well as GSSG increased gradually with increasing Cd^{2+} concentration in the nutrient solution.

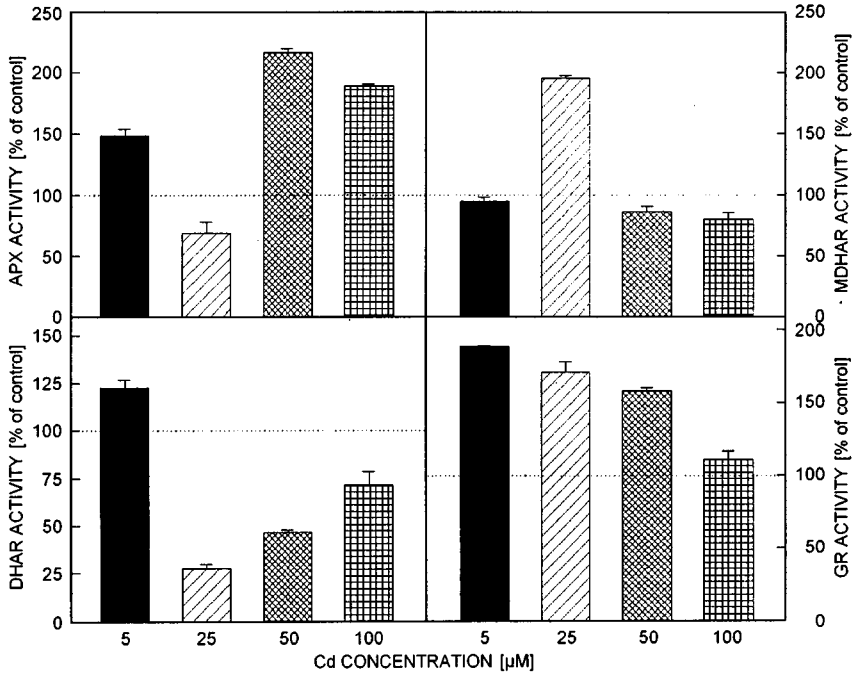

Fig. 2. Activity of the antioxidant enzymes in leaves of *Arabidopsis thaliana* growing for 7 d in the nutrient solution with different Cd concentration. In control plants activity of APX expressed as ΔA_{290} mg^{-1}(protein) min^{-1} was 3.34 ± 0.22. activity of MDHAR expressed as ΔA_{340} mg^{-1}(protein) min^{-1} was 0.19 ± 0.01; activity of DHAR expressed as ΔA_{265} mg^{-1}(protein) min^{-1} was 6.92 ± 0.21. activity of GR ΔA_{412} mg^{-1}(protein) min^{-1} was 0.85 ± 0.04.

Discussion

Mature plants of *Arabidopsis thaliana* growing even at the highest Cd^{2+} concentration in the nutrient solution did not show visual symptoms of metal toxicity. Cadmium was also accumulated more intensively in roots than in shoots of *Arabidopsis thaliana*, and in amounts increasing with its concentrations in the nutrient solution (Wójcik and Tukendorf 2000). However, after 7 d of *Arabidopsis thaliana* exposure to different Cd^{2+}

concentrations we observed in leaves some differences in the content of the individual radicals, *i.e.* various types of ROS dominated. At 5 μM Cd^{2+} the elevated $O_2^{\cdot-}$ content might be the result of the activity of NADPH-dependent superoxide synthase associated with the plasma membrane (Foyer *et al.* 1997) and a high content of H_2O_2 might be the product of enhanced total SOD activity. In the apoplast the presence of SOD (see Scandalios 1993) as well as ascorbate were found (see Horemans *et al.* 2000). At 5 μM Cd^{2+} an increase of SOD activity, strong accumulation of H_2O_2, lower activity of APX than POX and a high DHA level allow us to suppose that changes in the activity of the antioxidant system occur mainly in the apoplast.

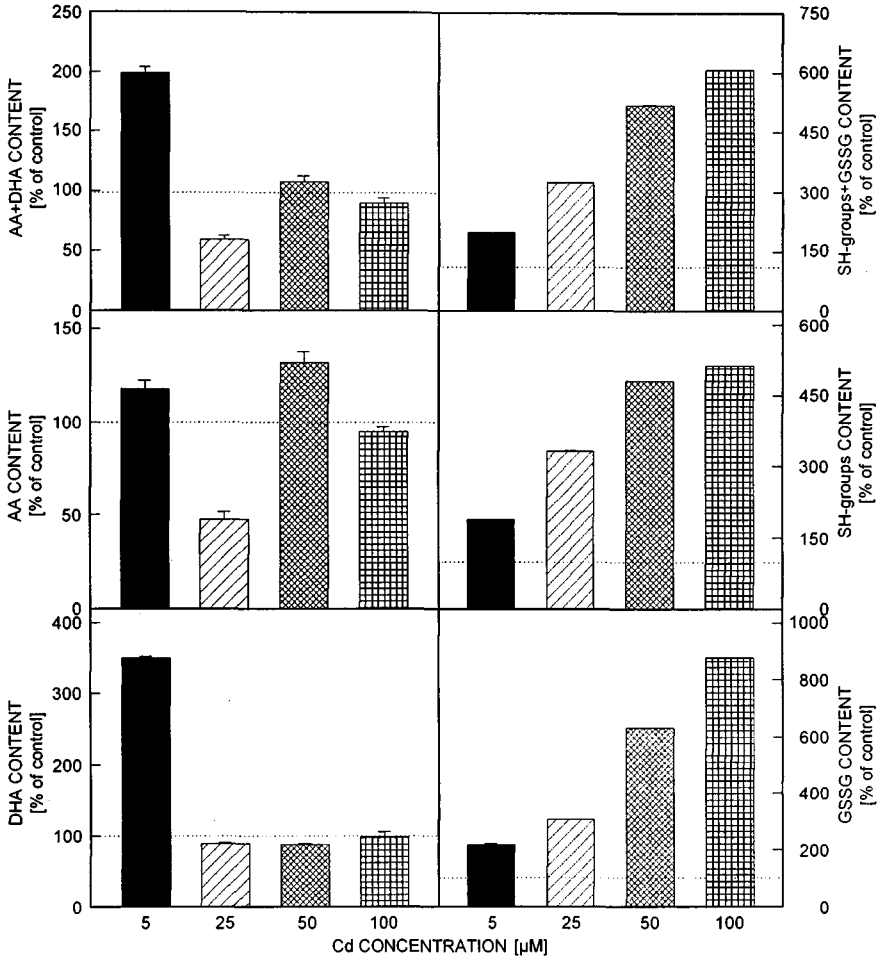

Fig. 3. The ascorbate, dehydroascorbate, SH groups and GSSG content in leaves of *Arabidopsis thaliana* growing for 7 d in the nutrient solution with different Cd concentration. In control plants the content of AA + DHA, AA and DHA in μmol g^{-1}(f.m.) was 5.83 ± 0.50, 3.77 ± 0.06, 2.06, respectively. In control plants the content of SH groups+GSSG, SH groups and GSSG in nmol g^{-1}(f.m.) was 193 ± 3.2; 142.6; 50.5 ± 4.2, respectively.

Differences in SOD activity are metal kind-, concentration- and plant-dependent (for details see Siedlecka *et al.* 2000). Total SOD activity increased in pea leaves infiltrated with 100 μM $CdCl_2$ (Dalurzo *et al.* 1997). The activity of SOD was not altered significantly in Cd^{2+}-treated seedlings of sugar cane, and at higher Cd^{2+} concentration, lack one of seven of Cu,Zn-SOD isoenzymes was observed (Fornazier *et al.* 2002).

At 25 μM Cd^{2+} in the nutrient solution SOD activity decreased in comparison with its activity at 5 μM Cd^{2+}, but it was still higher than control. In such stress conditions AA seems to play the main role in defence against a high content of $O_2^{\cdot-}$. Ascorbate can directly react with $O_2^{\cdot-}$ and H_2O_2 (Bartosz 1997), and MDHA, a semiquinone-like free radical, is formed as a result of the first AA oxidation. When the activity of MDHAR is high a large quantity of MDHA is reduced to AA, and its small amount remains available for disproportionation, therefore, DHA formation is low (Arrigoni 1994). A small content of AA, a high activity of MDHAR but a low activity of DHAR seem to confirm our supposition that at 25 μM Cd^{2+} ascorbate is directly responsible for ROS scavenging. Moreover, decreased AA content may act as a metabolic signal leading to increased GSH synthesis

(Arrigoni 1994). Elevated content of SH-groups and enhanced GR activity may indicate mobilization of an additional pathway of defence against the effect of heavy metals. In plants Cd^{2+} can be bound in non-toxic complex of phytochelatins – peptides, which are structurally related to GSH (Grill *et al.* 1985).

H_2O_2 is produced as a result of non-enzymatic and enzymatic reaction dismutation of $O_2^{\cdot-}$. However, stress situations exacerbate H_2O_2 production in the apoplast, in chloroplasts and in other cellular compartments as reported by Foyer *et al.* (1997). This kind of ROS is very mobile and can migrate to many cell compartments. Elevated ROS content leads to oxidative stress and the plant enzymatic antioxidant system is mobilised to remove its excess. However, it cannot be excluded that at 25 μM Cd^{2+} low activities of POX, APX, and CAT may be the result of a low content of their substrate - H_2O_2.

At 50 μM Cd^{2+} the $O_2^{\cdot-}$ amount was lower than in plants treated with 25 μM Cd^{2+}, but elevated $OH^{\cdot}$ content appeared. The activity of POX and APX was enhanced, H_2O_2 content was on the same level as in control and SOD activity was close to control. It may indicate that peroxidase catalyzes reaction which results in $OH^{\cdot}$ production from H_2O_2 in the presence of $O_2^{\cdot-}$, as shown by Chen and Schopfer (1999). An increase of POX activity was observed in Cd^{2+}-infiltrated leaves of *Pisum sativum* (Dalurzo *et al.* 1997) or when both older and young plants of *Phaseolus coccineus* were treated with Cd^{2+} (Skórzyńska-Polit *et al.* unpublished). Measurements of APX activity showed its increase under different heavy metal stress (Cakmak and Horst 1991, Shaw 1995, Chaoui *et al.* 1997, Weckx and Clijsters 1997, González *et al.* 1998, Navari-Izzo *et al.* 1998, Lidon and Teixeira 2000).

The increase of APX activity, elevated AA content and a high content of SH groups as well as GR activity in *A. thaliana* grown at 50 μM Cd^{2+} might indicate that the antioxidant system acting in chloroplasts and also other cellular compartments participated in response to oxidative stress. Further increase of SH group content in leaves of plants treated with 50 μM Cd in comparison with plants exposed to lower Cd^{2+} concentrations may prove that this kind of antioxidative defence system begins to dominate in *Arabidopsis thaliana*. It becomes more visible in plants treated with 100 μM Cd^{2+}, where SH group content is the highest. However, Xiang and Oliver (1998) showed that during 18 h of plant exposure to Cd phytochelatins appeared, the level of GSSG increased, and initially decreasing GSH level gradually returned to its initial level as the incubation was continued. They also showed that plants treated with Cd or Cu responded by increasing transcription of the gene for glutathione synthesis as well as for glutathione reductase. We observed an increase in SH-group content, but after 7 d of exposure to Cd^{2+} GR activity also enhanced. Moreover, Fornazier *et al.* (2002) observed a significant increase in GR activity after 4 d of seedlings exposure to $CdCl_2$.

A high content of $OH^{\cdot}$ indicates that elevated activities of enzymatic components of the antioxidant system are not enough efficient to prevent cells against ROS toxicity. Ascorbate was not sufficient for ROS scavenging. At higher Cd^{2+} concentrations in the nutrient solution metabolism of glutathione seems to be helpful in detoxification of the heavy metal in *Arabidopsis thaliana* plants.

In Cd^{2+}-treated *A. thaliana* plants POX activity dominates over catalase activity - another enzyme removing relatively long-living H_2O_2. On the one hand peroxidases have more affinity to H_2O_2 than catalase, and on the other catalase may be inhibited by $O_2^{\cdot-}$ and $OH^{\cdot}$. Catalase activity in *A. thaliana* decreased with increasing Cd concentration in the nutrient solution. In seedlings of sugar cane Cd^{2+} concentration-dependent decrease of CAT activity was also observed by Fornazier *et al.* (2002).

Heavy metals can change the activity of metabolic processes in cells in direct or indirect way and the plant response to lower concentrations of Cd^{2+} is different than to higher ones. At various Cd concentrations in the nutrient solution different levels of individual oxygen species are formed and dominate in leaves of *Arabidopsis thaliana*. In response to elevated content of ROS the antioxidant system is stimulated to prevent cells against damage. Our results show that the formation of the defence line in *A. thaliana* against heavy metal depends on its concentration. In *A. thaliana* a low Cd^{2+} concentration induces the activity of enzymatic antioxidant system but when its concentration is high an additional defence mechanism using GSH starts and it seems to play the pivotal role in detoxification of Cd^{2+}.

References

Aebi, H.: Catalase *in vitro*. - Methods Enzymol. **105**: 121-126, 1984.

Anderson, M.E.: Glutathione and glutathione disulfide in biological samples. - Methods Enzymol. **113**: 548-555, 1985.

Arrigoni, O.: Ascorbate system in plant development. - J. Bioenerg. Biomem. **26**: 407-419, 1994.

Babbs, C.F., Pham, J.A., Coolbaugh, R.C.: Lethal hydroxyl radical production in paraquat-treated plants. - Plant Physiol. **90**: 1267-1270, 1989.

Barceló, J., Poschenrieder, C.: Plant water relations as affected by heavy metal stress: a review. - J. Plant Nutr. **13**: 1-37, 1990.

Bartosz, G.: Oxidative stress in plants. - Acta Physiol. Plant. **19**: 47-64, 1997.

Beauchamp, C., Fridovich, I.: Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. - Anal.

Biochem. **44**: 276-287, 1971.

Boo, Y.C., Jung, J.: Water deficit - induced oxidative stress and antioxidative defences in rice plants. - J. Plant Physiol. **155**: 255-261, 1999.

Bradford, M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Cadenas, E.: Biochemistry of oxygen toxicity. - Annu. Rev. Biochem. **58**: 79-110, 1989.

Cakmak, I., Horst, W.J.: Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in roots tips of soybean (*Glycine max*). - Physiol. Plant. **83**: 463-468, 1991.

Chaoui, A., Mazhoudi, S., Ghorbal, M.H., El Ferjani, E.: Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L). - Plant Sci. **127**: 139-147, 1997.

Chen, S., Schopfer, P.: Hydroxyl-radical production in physiological reactions, A novel function of peroxidases. - Eur. J. Biochem. **260**: 726-735, 1999.

Dalurzo, H.C., Sandalio, L.M., Gómez, M., del Rio, L.A.: Cadmium infiltratration of detached pea leaves: effect on its activated oxygen metabolism. - Phyton **37**: 59-64, 1997.

Fornazier, R.F., Ferreira, R.R., Vitória, A.P., Molina, S.M.G., Lea, P.J., Azevedo, R.A.: Effects of cadmium on antioxidant enzyme activities in sugar cane. - Biol. Plant. **45**: 91-97, 2002.

Foyer, C.H., Lopez-Delgado, H., Dat, J.F., Scott, I.M.: Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. - Physiol. Plant. **100**: 241-254, 1997.

Gallego, S.M., Benavides, M.P., Tomaro, M.L.: Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. - Plant Sci. **121**: 151-159, 1996.

González, A., Steffen, K.L., Lynch, J.P.: Light and excess manganese, Implications for oxidative stress in common bean. - Plant Physiol. **118**: 493-504, 1998.

Green, M.J., Hill, H.A.O.: Chemistry of dioxygen. - Methods Enzymol. **105**: 3-22, 1984.

Grill, E., Winnacker, E.L., Zenk, M.H.: Phytochelatins: The principal heavy-metal complexing peptides of higher plants. - Science **230**: 674-676, 1985.

Horemans, N., Foyer, C.H., Asard, H.: Transport and action of ascorbate at the plant plasma membrane. - Trends Plant Sci. **5**: 263-267, 2000.

Knörzner, O.C., Durner, J., Böger, P.: Alterations in the antioxidative system of suspension-cultured soybean cells (*Glycine max*) induced by oxidative stress. - Physiol. Plant. **97**: 388-396, 1996.

Krupa, Z.: Cadmium against higher plant photosynthesis - a variety of effects and where do they possibly come from? - Z. Naturforsch. **54c**: 723-729, 1999.

Krupa. Z., Baszyński, T.: Some aspects of heavy metals toxicity towards photosynthetic apparatus - direct and indirect effects on light and dark reactions. - Acta Physiol. Plant. **17**: 177-190, 1995.

Lidon, F.C., Teixeira, M.G.: Oxy radicals production and control in the chloroplast of Mn-treated rice. - Plant Sci. **152**: 7-15, 2000.

Loggini, B., Scartazza, A., Brugnoli, E., Navari-Izzo, F.: Antioxidative defence system, pigment composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. - Plant Physiol. **199**: 1091-1099, 1999.

Mazhoudi, S., Chaoui, A., Ghorbal, M.H., Ferjani, E.E.: Response of antioxidant enzymes to excess copper in tomato (*Lycopersicon esculentum*, Mill.). - Plant Sci. **127**: 129-137, 1997.

Milosevic, N., Slusarenko, A.J.: Active oxygen metabolism and lignification in the hypersensitive response in bean. - Physiol. mol. Plant Pathol. **49**: 148-158, 1996.

Miyake, Ch., Asada, K.: Thylakoid-bound ascorbate peroxidase in spinach chloroplasts and photoreduction of its primary oxidation product monodehydroascorbate radicals in thylakoids. - Plant Cell Physiol. **33**: 541-553, 1992.

Nakano, Y., Asada, K.: Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate depleted medium and reactivation by monodehydroascorbate radical. - Plant Cell Physiol. **28**: 131-140, 1987.

Navari-Izzo, F., Quartacci, M.F., Pinzino, C., Vecchia, F.D., Sgherri, C.L.M.: Thylakoid-bound and stromal antioxidative enzymes in wheat treated with excess copper. - Physiol. Plant. **104**: 630-638, 1998.

Niewiadomska, E., Gaucher-Veilleux, C., Chevrier, N., Mauffette, Y., Dizengremel, P.: Elevated CO_2 does not provide protection against ozone considering the activity of several antioxidant enzymes in leaves of sugar maple. - J. Plant Physiol. **155**: 70-77, 1999.

Noctor, G., Foyer, C.H.: Ascorbate and glutathione: keeping active oxygen under control. - Annu. Rev. Plant Physiol. Plant mol. Biol. **49**: 249-279, 1998.

Pell, E., Schlanghaufer, C.D., Arteca, R.N.: Ozone-induced oxidative stress: Mechanism of action and reaction. - Physiol. Plant. **100**: 264-273, 1997.

Pick, E.: Microassays for superoxide and hydrogen peroxide production and nitroblue tetrazolium reduction using an enzyme immunoassay microplate reader. - Methods Enzymol. **132**: 407-421, 1986.

Prasad, M.N.V.: Trace elements. - In: Prasad, M.N.V. (ed.): Plant Ecophysiology. Pp. 207-249. John Wiley & Sons, New York 1996.

Rao, M.V., Hale, B.A., Ormrod, D.P.: Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. - Physiol. Plant. **109**: 421-432, 1995.

Rucińska, R., Waplak, S., Gwóźdź, E.A.: Free radical formation and activity of antioxidant enzymes in lupin roots exposed to lead. - Plant Physiol. Biochem. **37**: 187-194, 1999.

Salin, M.L.: Toxic oxygen species and protective systems of the chloroplast. - Physiol. Plant. **72**: 681-689, 1987.

Scandalios, J.G.: Oxygen stress and superoxide dismutases. - Plant Physiol. **101**: 7-12, 1993.

Schickler, H., Caspi, H.: Response of antioxidative enzymes to nickel and cadmium stress in hyperaccumulator plants of the genus *Alyssum*. - Physiol. Plant. **105**: 39-44, 1999.

Shaw, B.P.: Effect of mercury and cadmium on the activities of antioxidative enzymes in the seedlings of *Phaseolus aureus*. - Biol. Plant. **37**: 587-596, 1995.

Siedlecka, A., Krupa, Z., Tukendorf, A. Skórzyńska-Polit, E., Maksymiec, W., Wójcik, M., Baszyński, T.: Angiosperms (Asteraceae, Convolvulaceae, Fabaceae and Poaceae: other than Brassicaceae). - In: Prasad, M.N.V. (ed.): Metals in the Environment: Analysis by Biodiversity. Pp. 171-217. Marcel Dekker, New York - Basel 2000.

Sies, H.: Strategies of antioxidant defence. - Eur. J. Biochem. **215**: 213-219, 1993.

Smirnoff, N., Colombé, S. Drought influences the activity of

enzymes of the chloroplast hydrogen peroxide scavenging system. - J. exp. Bot. **39**: 1097-1108, 1988.

Stroiński, A.: Some physiological and biochemical aspects of plant resistance to cadmium effect. I. Antioxidative system. - Acta Physiol. Plant. **21**: 175-188, 1999.

Vácha, F.: The role of oxygen in photosynthesis. - Photosynthetica **31**: 321-334, 1995.

Wang, S.Y., Jiao, H.J., Faust M.: Changes in ascorbate, glutathione, and related enzyme activities during thidiazuron-induced bud break of apple. - Physiol. Plant. **82**: 231-236, 1991.

Weckx, J.E.J., Clijsters, H.M.M.: Oxidative damage and defence mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. - Physiol. Plant. **96**: 506-512, 1996.

Weckx, J.E.J., Clijsters, H.M.M.: Zn phytotoxicity induces oxidative stress in primary leaves of *Phaseolus vulgaris*. - Plant Physiol. Biochem. **35**: 405-410, 1997.

Wójcik, M., Tukendorf, A.: Cd-tolerance of wild type of *Arabidopsis thaliana*. - Plant Physiol. Biochem. **38** (Suppl.): 181, 2000.

Woźny, A., Stroiński, A., Gwóźdź, E.: Plant Cell Responses to Cadmium. - Wydawnictwo Naukowe Uniwersytetu Im. Adama Mickiewicza, Poznań 1990.

Xiang, C., Oliver, D.J.: Glutathione metabolic genes coordinatelly responded to heavy metals and jasmonic acid in *Arabidopsis*. - Plant Cell **10**: 1539-1550, 1998.