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Effect of aluminium on oxidative stress related enzymes activities in barley roots

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Abstract

The impact of aluminium stress on activities of enzymes of the oxidative metabolism: superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), NADH peroxidase (NADH-POD) and oxalate oxidase (OXO) was studied in barley (*Hordeum vulgare* L. cv. Alfor) root tips. SOD appeared to be involved in detoxification mechanisms at highly toxic Al doses and after long Al exposure. POD and APX, H_2O_2 consuming enzymes, were activated following similar patterns of expression and exhibiting significant correlation between their elevated activities and root growth inhibition. The signalling role of NADH-POD in oxidative stress seems to be more probable than that of OXO, which might be involved in Al toxicity mechanism.

Additional key words: ascorbate peroxidase, *Hordeum vulgare*, NADH peroxidase, oxalate oxidase, peroxidase, superoxide dismutase.

Introduction

Aluminium toxicity became a factor limiting crop productivity on acid soils. Al is supposed to alter the plasma membrane properties by enhancing the peroxidation of phospholipids and proteins (Cakmak and Horst 1991), alter the cation-exchange capacity of the cell wall (Horst 1995), interfere with signal transduction (Jones and Kochian 1995), binds directly to DNA or RNA, *etc*. Various mechanisms of Al tolerance have been suggested. Organic acids such as malate, citrate, and oxalate exuded into the rhizosphere can prevent roots by chelating Al (Ryan *et al.* 1995). Similar strategy has been reported in Al-accumulating plants detoxifying Al by internal chelating with citrate (Ma *et al.* 1997) or oxalate (Ma *et al.* 1998). Al stress induces the expression of a number of genes; over 20 of them have already been isolated and characterized in wheat (Snowden *et al.* 1995, Cruz-Ortega *et al.* 1997, Hamilton *et al.* 2001), tobacco (Ezaki *et al.* 1995, 1997) and *Arabidopsis* (Richards *et al.* 1998) and their hypothetical functions in Al toxicity or Al resistance mechanisms have been proposed. Since some of these Al-induced genes are coding for antioxidant enzymes (glutathion S-transferase, peroxidase, superoxide dismutase), it has been suggested that there is a strong connection between Al stress and oxidative stress in plants (Cakmak and Horst 1991, Richards *et al.* 1998). Ezaki *et al.* (2000) confirmed this hypothesis when they showed that overexpression of some Al-induced genes in transgenic *Arabidopsis* plants conferred oxidative stress resistance. The key role of antioxidant enzymes is to reduce or scavenge reactive oxygen species (ROS) such as superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radical. Superoxide dismutases (SOD, EC 1.15.1.1), located in various cell compartments, constitute the first line of defense against ROS. They convert superoxide to H_2O_2 and O_2 and therefore offer the protection against superoxide-induced oxidative stress. The resulting H_2O_2 is removed through the activity of Asada-Halliwell scavenging cycle in chloroplasts and cytoplasm by ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase activities (Asada 1992). Peroxidases (POD, EC 1.11.1.7.) participate in lignin biosynthesis, cell wall cross-linkage, IAA degradation, disease resistance, and convert H_2O_2 to water (Asada 1992, Siegel 1993). A number of potential sources of

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Abbreviations: ROS - reactive oxygen species, APX - ascorbate peroxidase, NADH-POD - NADH peroxidase, OXO - oxalate oxidase, PM - plasma membrane, POD - peroxidase, SOD - superoxide dismutase.

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ROS can be found in plant cells. One of the mechanisms leading to H_2O_2 production consists in oxidation of NADH catalysed by a cell wall peroxidase (NADH-POD) (Mäder *et al.* 1980). An alternative source, oxalate oxidase (OXO, EC 1.2.3.4.), produces H_2O_2 by oxidation of oxalate.

The objective of this study was to evaluate the effect of Al on the activities of five enzymes of oxidative metabolism (superoxide dismutase, ascorbate peroxidase, peroxidase, oxalate oxidase, and NADH peroxidase) involved either in production or in degradation of ROS in roots of barley cultivar Alfor.

Materials and methods

Plants, growth, and stress conditions: Caryopses of barley (*Hordeum vulgare* L., cv. Alfor) were surface sterilized with 12 % H_2O_2 for 10 min and then rinsed five times with distilled water. After incubating in distilled water for 4 h at 24 °C in darkness, the caryopses were transferred to the filter paper (*Whatman No. 1*) moistened either in 0.2 mM $CaCl_2$, pH 4.5 (control), or with $AlCl_3 . 6 H_2O$ (concentrations ranging from 2 to 8 mM) in 0.2 mM $CaCl_2$, pH 4.5 (Al-treated). Millimolar concentrations of Al were applied due to the high Al binding capacity of filter paper used for incubation. Following incubation at 24 °C in darkness continued 48 or 72 h. The collected root tips (1 cm) were stored at -70 °C until analyzed. All experiments were repeated three times.

Determination of cell death: The loss of plasma membrane (PM) integrity was evaluated by Evans blue staining (Baker and Mock 1994). Roots were stained in 0.25 % (v/v) aqueous solution of Evans blue for 15 min at room temperature and washed three times with distilled water, each for 10 min. Excised root tips (0.5 cm) were soaked in N,N-dimethylformamide for 24 h at 4 °C. Absorbance was measured at 600 nm (spectrophotometer *Beckman DU-8B*, Salzburg, Austria).

Protein extraction and enzyme extracts preparation: Root tips were ground to a fine powder in a cold mortar in liquid nitrogen and the resulting powder was rehomogenised in 40 mM succinic acid/NaOH, buffer, pH 4.0, with homogenisator (*Heidolph DIAX 900*, Kolhaim, Germany). The homogenate was filtered and centrifuged at 1 500 *g* for 5 min, then at 12 000 *g* for 15 min and finally at 150 000 *g* for 30 min (*Beckman L8-M*, Palo Alto, USA). The resulting supernatant was used for analysis. Protein content was measured according to the method of Bradford (1976), with bovine serum albumin (BSA) as a standard.

Enzyme assays: Determination of the activity of SOD was based on the method of Beyer and Fridovich (1987). The reaction mixture was composed of 15 μM methionine, 70 μM 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 20 μM riboflavin, potassium phosphate buffer (0.05 M, pH 7) and appropriate volume of root extract. The reaction was initiated by illumination. The blue formazane produced by MTT photoreduction was measured at 560 nm. APX activity was determined by following the decrease of absorbance at 290 nm. The reaction mixture contained 0.5 mM ascorbate, 0.2 mM hydrogen peroxide, 0.05 M potassium phosphate (pH 7.0) and the suitable volume of root extract (Chen and Asada 1989). The activity of POD was determined by monitoring the formation of guaiacol dehydrogenation product by following the increase of absorbance at 405 nm, by the method of Chance and Maehly (1955). Reaction mixture contained 0.04 % guaiacol, sodium 0.04 M acetate buffer (pH 5.2) and the suitable volume of root extract. The reaction was initiated by adding 0.04 % hydrogen peroxide. OXO activity was determined by the method of Zhang *et al.* (1996). The reaction mixture contained 40 mM succinic acid/NaOH buffer, pH 4.0, 60 % ethanol (v/v), 2 mM oxalic acid, 0,02 % N, N-dimethylaniline, 0,4 mM 4-aminoantipyrine and 5 units cm^{-3} of horseradish peroxidase. The mixture was incubated at 37 °C for 15 min and activity was measured at 555 nm against the control reaction without oxalic acid. The activity of NADH–POD was determined by following the decrease of absorbance at 340 nm by oxidizing NADH. The reaction mixture contained 0.15 mM NADH, 0.2 mM hydrogen peroxide, sodium phosphate buffer (0.05 M, pH 7) and the suitable volume of plant extract (De Marco and Roubelakis-Angelakis 1996).

The Student *t*-test was used to establish the significance of differences between means of control and treated plants.

Results

Root growth inhibition and cell death: One of the very early symptoms of Al toxicity is root growth inhibition, which can be accompanied by cell death as a consequence of the loss of PM integrity at higher Al concentrations. To examine the relation between the root growth inhibition, cell death and activation of five

enzymes of oxidative metabolism, barley caryopses were treated with Al in a range 0 - 8 mM for 48 and 72 h. After 48 h of Al treatment, root growth was proportionally inhibited with concentrations higher than 2 mM Al, and at 8 mM Al root length represented only 39 % that of control roots (Fig. 1*A*). Loss of PM integrity, detected by Evans blue uptake, also continuously rose up from 2 to 8 mM Al (Fig. 1*B*). After 72 h of Al treatment, root growth was significantly inhibited already at 2 mM Al (Fig. 1*A*) and rose up to 8 mM where the root length of Al-treated seedlings represented only 20 % that of control roots. At this concentration high increase of Evans blue uptake was also detected. These results indicate positive correlation between the loss of PM integrity and root growth inhibition in relation to Al concentration.

Enzymes activities: To examine the activation of five oxidative stress-related enzymes the same Al concentration range was applied and Al dose dependency of enzymes expression was evaluated after 48 and 72 h. After 48 h of Al exposure at 2 mM only slight activation of NADH-POD was exhibited (Fig. 2*E*). No significant induction of other oxidative stress-related enzymes could be detected. Increase in Al concentration to 4 mM caused only minor increase in POD activity (Fig. 2*C*). The exposure of barley roots to 6 and 8 mM Al caused significant increase in APX, POD and NADH-POD activity (Fig. 2*B*,*C*,*E*), positively correlated with loss of PM integrity and root growth inhibition. Protective mechanisms against Al-stress seem to be conferred by the activation of SOD, APX, POD and NADH-POD (Fig. 2*A*,*B*,*C*,*E*). 72-h treatment at 4 mM Al increased the activity of SOD, APX, POD and NADH-POD (Fig. 2*A*,*B*,*C*,*E*) in comparison to 2 mM. At 6 and 8 mM Al OXO activity (Fig. 2*D*) was increased.

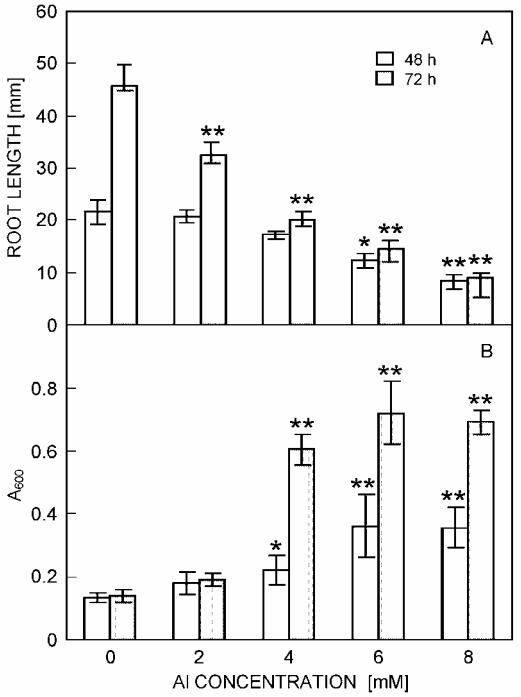

Fig. 1. Root growth inhibition (*A*) and loss of PM integrity evaluated by Evans blue staining method (*B*) after 48 h and 72 h of 0, 2, 4, 6 and 8 mM Al treatment. Means ± SD of three experiments. According to Student´s *t*-test, means denoted by * and ** are significantly different from the appropriate control at $P \leq 0.05$ and 0.01 levels, respectively.

Discussion

Increased SOD activity suggests an elevated content of superoxide radical; especially at concentrations where root growth was strongly inhibited and PM integrity lost. No changes in SOD activity after 48 h and moderate increase in comparison to APX or POD activities after 72 h might be explained by high basal SOD activity in control plants. Aluminium was shown to enhance SOD activity in root tips of soybean (Cakmak and Horst 1991), roots of *Arabidopsis* (Richards *et al*. 1998), and roots of sorghum (Peixoto *et al.* 1999). Lee *et al.* (2001) suggested that enhanced activity of SOD may function in signaling of oxidative stress, which leads to the induction of antioxidant enzymes associated with an H_2O_2 scavenging system, particularly an ascorbate-glutathione cycle. Since in our experiments SOD activity was increased only after 72 h of Al treatment, the signaling role in induction of other antioxidative enzymes, activity of which increased as soon as after 48 h, could not be confirmed.

The increase in APX activity indicated elevated Al-enhanced H_2O_2 production in root tissues. Although the actual H_2O_2 concentration has not been measured, this assumption was supported also by increased activities of NADH-POD and OXO, H_2O_2 producing enzymes, especially at 6 and 8 mM Al after 72 h. It has been previously suggested that accumulation of H_2O_2 caused by various environmental stresses would result in the enhanced activity of APX and catalase in order to protect plant cells (Mizuno *et al.* 1988). Dramatic enhancement of APX activity in comparison to POD activity in barley roots supplied by Cd (Hegedüs *et al.* 2001) led authors to the assumption that ROS detoxifying mechanism operates rather with APX than with POD.

On the contrary, our results showed substantially higher increase in activity of POD than of APX at any Al concentration applied. The role of POD in Al stress response might consist in scavenging the toxic lipid hydroperoxides generated by the peroxidation of

membrane lipids, as suggested by Ezaki *et al.* (1996). These authors proposed, that peroxidase isozymes expressed in the soluble fraction of Al-stressed tobacco cells might detoxify peroxides generated by Al intruded through the membrane into the cytosol. Hegedüs *et al.* (2001) suggested an alternative role of POD isozymes participating in lignin biosynthesis to build up the physical barrier against toxic metals entering the cell. The role of peroxidases in control of cell elongation has also been proposed (Lagrimini *et al.* 1997, Souza and MacAdam 2001). Our experiments confirmed a significant correlation between elevated POD activity and inhibition of root growth under Al stress. Similarly, the root growth inhibition caused by salinity stress was suggested to be associated with cell-wall stiffening catalyzed by peroxidase activity in dimerization of ferulic acid (Lin and Kao 2001).

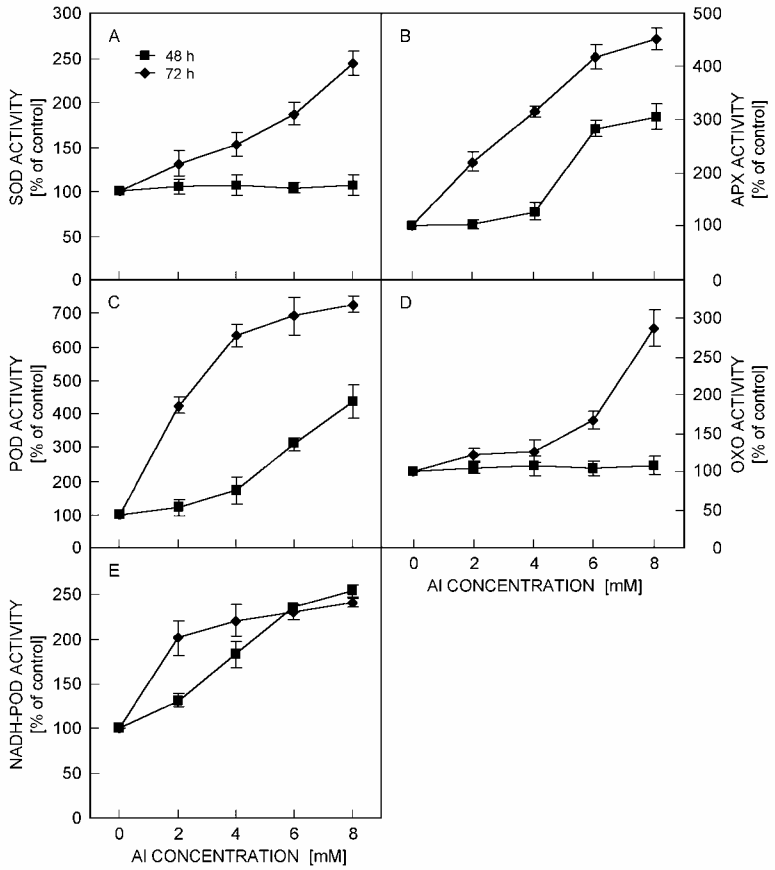

Fig. 2. SOD activity (*A*), APX activity (*B*), POD activity (*C*), OXO activity (*D*) and NADH-POD activity (*E*) after 48 h and 72 h of 0, 2, 4, 6 and 8 mM Al treatment. Changes in enzyme activities are expressed as a percentage of control. Means ± SD of three experiments. Specific enzyme activities in control treatments after 48 h: SOD: 0.0388 ± 0.0047 ΔA_{560} min^{-1} µg^{-1}(protein), APX: 0.0061 ± 0.0006 ΔA_{290} µg^{-1}(protein) min^{-1}, POD: 0.9066 ± 0.2862 ΔA_{405} µg^{-1}(protein) min^{-1}, OXO: 0.0013 ± 0.0007 ΔA_{555} µg^{-1}(protein) min^{-1}, NADH-POD: 0.0093 ± 0.0024 ΔA_{340} µg^{-1}(protein) min^{-1}, and after 72 h: SOD: 0.0421 ± 0.0053 ΔA_{560} µg^{-1}(protein) min^{-1}, APX: 0.0033 ± 0.0013 ΔA_{290} µg^{-1}(protein) min^{-1}, POD: 0.9132 ± 0.0928 ΔA_{405} µg^{-1}(protein) min^{-1}, OXO: 0.0012 ± 0.0006 ΔA_{555} µg^{-1}(protein) min^{-1}, NADH-POD: 0.0056 ± 0.0013 ΔA_{340} µg^{-1}(protein) min^{-1}.

Increased activity of POD was also described in soybean roots treated with Al (Cakmak and Horst 1991). Ezaki *et al.* (2000) suggested that the enhanced activity of anionic POD could act in conferring Al resistance by detoxifying ROS and restricting lipid peroxidation in membrane regions. In contrast, Jan *et al.* (2001) proposed the increased POD activity to be part of a damage response to Al, since they showed that Al induced POD activities in Al-sensitive rice cultivar whereas in Al-tolerant cultivar they were unaffected by Al treatment. Similarly, increased POD was suggested to reflect the damage response to salinity in rice (Mittal and Dubey 1991).

According to our results, it appears that OXO expression might be required as H_2O_2 producing enzyme in Al induced cell death process. Strong correlation between OXO activation and detection of cell death was found at 6 and 8 mM after 72 h of Al treatment. The expression of OXO only at high Al concentration and the correlation with cell death induction could indicate that

OXO-induced H_2O_2 production might be related to Al toxicity mechanism occurring probably by necrosis. On the other hand, Delisle *et al.* (2001) proposed that Al-induced OXO expression in wheat roots could be involved in detoxification mechanism. Authors observed small groups of death cells only in the root of Al-treated tolerant wheat cultivar and suggested that accelerated epidermal cell turnover maight represent a detoxification mechanism helping to protect deeper root cell layers. OXO-like proteins or mRNA were also shown to accumulate in response to fungal infection (Zhang *et al.* 1995, Dumas *et al.* 1995), where OXO acts in the local provision of H_2O_2 for cross-linking of cell wall components in infection sites in a process of hypersensitive response. OXO was shown to be stimulated also in barley roots by salt stress (Hurkman and Tanaka 1996).

The actual function of NADH-POD, a H_2O_2 producing enzyme in plants is as yet unclear, but it has been suggested that it might act in providing H_2O_2 for other peroxidase isozymes (Ezaki *et al.* 1996). According to our results, we suggest that NADH-POD could act in early signalling of oxidative stress through producing H_2O_2 since it was activated as soon as after 48 h and at low Al concentrations. An alternative role of NADH-POD in cell wall loosening was suggested by Chen and Schopfer (1999). Cell wall loosening, mediated by H_2O_2 induced cross-linking of cell wall polymers, might compensate the increase of cell wall rigidity induced by Al. NADH POD activity and H_2O_2 formation were necessary to allow proper cell wall reconstruction of regenerating protoplast (De Marco and Roubelakis-Angelakis 1996).

To conclude, root growth inhibition was positively correlated with the loss of PM integrity. SOD appears to be involved in detoxification mechanisms at highly toxic Al doses and after longer exposure of roots to Al. POD and APX, H_2O_2 consuming enzymes, were activated following similar patterns of expression and exhibiting significant correlation between their elevated activities and root growth inhibition. The signalling role of NADH-POD in Al stress seems to be more probable than that of OXO, which might be involved in Al perception mechanism.

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