Changes in the ascorbate system in the response of pumpkin (*Cucurbita pepo* L.) roots to aluminium stress

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Abstract

The involvement of the ascorbate (AsA) system in the response of pumpkin (*Cucurbita pepo* L.) roots to aluminium stress was studied. The treatment of 5-day-old pumpkin seedlings with 50 μM aluminium sulphate resulted in approximately 60% inhibition of root growth within 48–60 h of treatment, while aluminium accumulated in the roots reaching a maximum within 48 h. During the same period, the hydrogen peroxide content of the roots was strongly enhanced. The increased level of hydrogen peroxide was matched by both increased ascorbate peroxidase (APX) (EC 1.11.1.11) activity and ascorbate free radical reductase (AFRR) (EC 1.1.5.4) activity, while dehydroascorbate reductase (DHAR) (EC 1.8.5.1) and glutathione reductase (GR) (EC 1.6.4.2) did not change. The levels of AsA in the roots were also increased by the Al treatment. It was concluded that an oxidative burst is probably involved in the toxicity of Al in pumpkin roots and that plants react to the enhanced production of reactive oxygen species by expressing higher levels of scavenging systems such as the AsA–APX system.
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Introduction

Aluminium is a major constituent of soil and, consequently, plants grow in soil environments in which the roots are potentially exposed to high concentrations of aluminium. Fortunately, Al precipitates at alkaline, neutral, or mildly acid soil pH in the form of insoluble salts. However, when soil pH decreases to 5 or lower, soluble forms of Al can accumulate to concentrations that inhibit root growth and function. The most toxic Al species seems to be Al^{3+}, although a very toxic polynuclear complex, which is referred to as Al_{13}, has been shown to be formed in partially neutralized solutions (Kinraide, 1991). Al toxicity is one of the major factors limiting crop production on acid soils and, since a large part of the world's total land area consists of acid soil, much attention has been paid to Al toxicity and resistance (for a review see Kochian, 1995; Delhaize and Ryan, 1995; Matsumoto, 2000).

The first evident symptom of Al toxicity is the inhibition of root growth, which can occur after very brief exposure of roots to Al. Although many studies have focused on this aspect of toxicity and various mechanisms for it have been proposed, the reasons for Al toxicity are still poorly understood. The primary site of Al accumulation and toxicity has been shown to be the root meristem, suggesting that Al interacts with actively dividing and elongating cells (Delhaize and Ryan, 1995). Many different mechanisms have been hypothesized to explain Al toxicity. The induction of callose synthesis in response to Al toxicity has been reported as a consequence of Al interaction with the root cell wall (Massot et al., 1999). Other research has demonstrated that important Al-induced events are associated with the plasma membrane. Since Al can rapidly reach the cytosol (Silva et al., 2000), it probably affects physiological parameters such as membrane potential, ion fluxes, and signal transduction pathways (Piñeros and Kochian, 2001). For instance, Al is able to block Ca^{2+} channels at the plasma membrane of cultured tobacco cells (Jones et al., 1998).

Many studies also concern Al resistance in plants. Sequestration into the vacuole might be a mechanism of true tolerance (Vázquez et al., 1999). However, exudation into the rhizosphere of Al-chelating organic acids, such as malate, oxalate, and citrate, seems to play a more important role in avoiding Al toxicity (Yang et al., 2000).

Furthermore, many studies have involved Al-induced genes, most of which are also expressed during oxidative stress, pathogen interaction, heat shock, and other different stresses. However, the exact role of Al-induced genes in Al stress has not yet been elucidated.

Richards et al. (1998) reported that oxidative stress genes, including peroxidase and glutathione-S-transferase, were induced in *Arabidopsis thaliana* in the presence of aluminium. The possible connection between Al stress and oxidative stress had also been previously suggested by Cakmak and Horst (1991) following the finding that the Al-induced inhibition of root elongation was correlated with enhanced lipid peroxidation. Moreover, superoxide dismutase and peroxidase activity were found to increase while catalase decreased. These findings were interpreted in the sense that the primary effect of Al could be the induction of free radical generation and related alterations in membrane structure, although the same authors subsequently reported that membrane disruption could not be considered a short-term response of soybean roots to aluminium (Horst et al., 1992).

Further evidence corroborating the relation between aluminium stress and oxidative stress in plants has recently been obtained with transgenic *Arabidopsis* plants (Ezaki et al., 2000). However, since Al is not a transition metal, it cannot by itself catalyze redox reactions. It has been suggested that Al indeed sensitizes membranes to an Fe-mediated free-radical chain reaction leading to lipid peroxidation (Yamamoto et al., 2001).

Oxidative stress is characterized by the production of reactive oxygen species (ROS) that are able to initiate a free radical chain reaction. Both plants and animals possess antioxidant systems that counteract the action of ROS. Among these antioxidant systems one of the most important is the ascorbate (AsA) system, which has been well studied in relation to both abiotic and biotic stresses. Abiotic conditions that have been studied include, among others, low temperature (Walker and McKersie, 1993), herbicide challenge (Iturbe-Ormaetxe et al., 1998), drought (Mittler and Zilinskas, 1994), wounding (Grantz et al., 1995), SO_2 fumigation (Kubo et al., 1995), and ozone exposure (Ranieri et al., 1996). Moreover, a relationship between Al toxicity and AsA metabolism has been reported in summer squash (Lukaszewski and Blevins, 1996).

In this study we investigated whether the AsA system is modified in pumpkin roots in response to Al treatment.

Materials and methods

Plant material and growth conditions

Pumpkin seeds (*Cucurbita pepo* L. var. Butternut) were surface sterilized in 1% (v/v) sodium

hypochlorite for 15 min, and then repeatedly washed with sterile distilled water. Washed seeds were germinated on wet filter paper in Petri dishes for 5 days in the dark. Five-day-old seedlings showed well-developed roots (approximately 2 cm in length), and a short hypocotyl. Al treatments were performed by bathing the roots of 5-day-old seedlings in solutions containing $Al_2(SO_4)_3$. Both treatment and control solutions were supplemented with 100 μM $CaCl_2$ according to Yamamoto et al. (2001) and the pH was adjusted to 4.5. Solutions were frequently renewed to prevent pH changes.

Determination of aluminium

For the determination of the Al content of the root tips, 50 mg samples of root tips (5 mm in length) were dried at 70 °C and burned to ashes in a muffle furnace at 550 °C overnight. Ashes were suspended in 6 N HCl and analysed for Al and Ca content using an inductively coupled plasma atomic emission spectrometer (ICP–AES), model "trace scan"– Sequential Thermo Jarrell Ash.

Histochemical analyses were also carried out both to localize Al in the roots and to assess the loss of integrity of root cell membranes. The localization of Al was detected with haematoxylin according to Sasaki et al. (1997). The loss of plasma membrane integrity was revealed by staining with Evans blue according to Yamamoto et al. (2001).

Determination of hydrogen peroxide

The H_2O_2 contents of both control and treated roots were determined according to Loreto and Velikova (2001). Approximately 100 mg of root tips, 5 mm long, were homogenized at 4 °C in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000g for 15 min and 0.5 ml of the supernatant were mixed with 0.5 ml of 10 mM potassium phosphate buffer pH 7.0 and 1 ml of 1 M KI. The H_2O_2 content of the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve.

Enzyme assays

Approximately 300 mg of pumpkin root tips, 5 mm long, was frozen in liquid nitrogen and then homogenized in cold 50 mM Tris buffer pH 8.0 containing 0.3 M mannitol, 1 mM EDTA, 10 mM $MgCl_2$, 0.05% cystein. The buffer/tissue ratio was 3:1. The homogenate was centrifuged at 22,000g for 20 min and the supernatant was used for enzyme analyses.

Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was determined as described by De Leonardis et al. (2000) by measuring the decrease in absorbance at 290 nm in a reaction mixture containing 50 mM phosphate buffer pH 7.0, 500 μM AsA, 1 mM H_2O_2 and 100 μl enzyme extract. The extinction coefficient of AsA was 2.8 mM^{-1} cm^{-1}.

Dehydroascorbate reductase (DHAR) (EC 1.8.5.1) activity was determined as described by Dipierro and Borraccino (1991). The reaction mixture contained 200 mM phosphate buffer pH 6.2 containing 4 mM dehydroascorbate, 2 mM reduced glutathione, and 100 μl enzyme extract. The extinction coefficient of AsA was 14 mM^{-1} cm^{-1} at 265 nm.

Ascorbate free radical reductase (AFRR) (EC 1.1.5.4) was measured according to the method of De Leonardis et al. (1995). The reaction mixture contained 100 mM Tricine/NaOH pH 8.0, 200 μM NADH, 1 mM AsA, 100 μl enzyme extract and 3 μg ascorbate oxidase.

Glutathione reductase (GR) (EC 1.6.4.2) was measured according to the method of Rao (1992). The assay contained 200 mM phosphate buffer pH 7.0, 2 mM EDTA, 100 μM NADPH, 1 mM oxidized glutathione, and 300 μl enzyme extract.

For both AFRR and GR the extinction coefficient of NAD(P)H at 340 nm was 6.22 mM^{-1} cm^{-1}.

The protein content of extracts was determined according to Bradford (1976).

Determination of AsA and dehydroascorbate

Approximately 300 mg of root tips (wet weight) was frozen with liquid nitrogen, ground in a mortar with 3 ml of 5% metaphosphoric acid, and centrifuged at 20,000g for 15 min. The pellet was discarded and the supernatant used for the determination of AsA and dehydroascorbate (DHA) as described by Kampfenkel et al. (1994). The assay was based on the reduction of Fe^{3+} to Fe^{2+} by AsA and the spectrophotometric detection of Fe^{+2} complexed with 2,2′-dipyridyl. DHA was reduced to AsA by preincubating the sample with dithiothreitol (DTT). The excess DTT was removed with N-ethylmaleimide, and the total AsA was determined. The amount of DHA was the difference between total AsA and the AsA measured prior to the DTT treatment.

Results

Aluminium effects on root growth

After 5 days of germination in the dark, the roots of pumpkin seedlings were, on average, 20 ± 5 mm

long. The treatment of these pumpkin seedlings with increasing concentrations of aluminium resulted in a dose-dependent inhibition of root growth. The inhibition increased up to approximately 60% at 50 μM aluminium sulphate. No significant further increase in inhibition was observed at higher aluminium concentrations (Fig. 1A). Percent inhibition was calculated according to Yamamoto et al. (2001). Maximum inhibition was achieved within 48–60 h of treatment with 50 μM aluminium sulphate (Fig. 1B). Staining the treated roots with haematoxylin showed that aluminium was localized mainly in the apical region as far as 5 mm from the tip. The same region retained Evans blue, thus indicating that membranes had been damaged. Therefore, all further experiments were carried out with apical segments, which were 5 mm long. Root tips of seedlings bathed in 50 μM aluminium sulphate accumulated high amounts of aluminium as revealed by ICP–AES (Table 1). The accumulated amount reached a maximum within 48 h and then decreased. The increased aluminium

content of the segments was matched by decreased calcium content. This finding was in agreement with other reports in the literature (Jones et al., 1998). Table 1 also shows that the amounts of ashes obtained from different samples were of the same order of magnitude. However, the percent aluminium in ashes was higher in treated than in control samples. Therefore, we conclude that aluminium interfered with the uptake of other mineral elements.

Production of hydrogen peroxide and activity of scavenging systems

The loss of membrane integrity that became evident by staining roots with Evan's blue was matched by an increased production of hydrogen peroxide in Al-treated roots. The level of H_2O_2 measured in treated segments was higher than that of control segments and reached a maximum within 48 h (Fig. 2). Since Al accumulation in the roots also
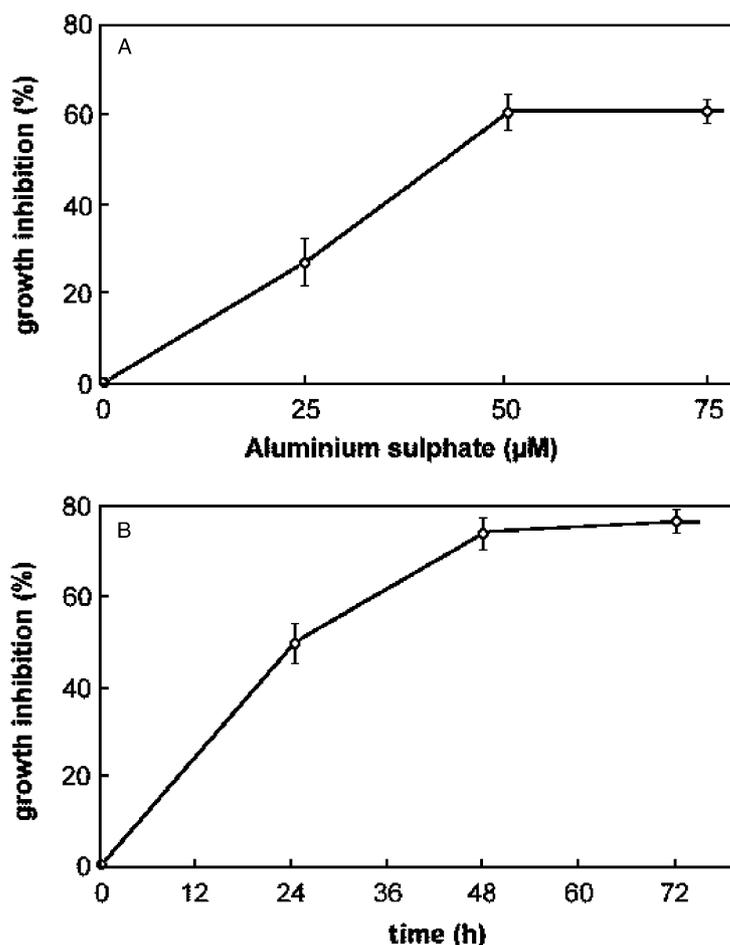
Figure 1. Percent growth inhibition of the roots of 5-day-old pumpkin seedlings treated with aluminium sulphate: (A) dose–response relationship and (B) time course of the inhibition induced by treatment with 50 μM aluminium sulphate. Values represent the means ($\pm$SE) of three experiments.

Table 1. Effect of 50 μM aluminium sulphate on the Al and Ca contents of pumpkin roots. Five-day-old seedlings were bathed for up to 72 h with 50 μM $Al_2(SO_4)_3$ in 100 μM $CaCl_2$ and 50 mg samples of root tips, 5 mm long, were analyzed for Al and Ca contents after 24, 48 and 72 h of treatment (C=control, T=treated). Values represent mean $\pm$ SE of three experiments.

Sample	d.w. (mg)	% Ash	Al (mg/kg d.w.)	% Al in ash	Ca (mg/kg d.w.)	% Ca in ash
C_{24}	11.8 ± 1.0	11.02 ± 0.97	148 ± 13	0.16 ± 0.01	4348 ± 178	3.91 ± 0.16
T_{24}	14.8 ± 0.8	7.43 ± 0.38	7095 ± 365	9.55 ± 0.49	3378 ± 162	4.52 ± 0.22
C_{48}	48.6 ± 3.9	16.8 ± 1.34	200 ± 16	0.13 ± 0.01	3845 ± 145	4.01 ± 0.15
T_{48}	60.2 ± 3.0	13.6 ± 0.69	15600 ± 793	11.5 ± 0.58	1700 ± 80	2.10 ± 0.10
C_{72}	20 ± 2.2	9.50 ± 1.05	162 ± 18	0.17 ± 0.02	2725 ± 128	2.89 ± 0.14
T_{72}	35.8 ± 1.9	9.50 ± 0.51	5940 ± 321	6.25 ± 0.33	3771 ± 123	4.04 ± 0.13
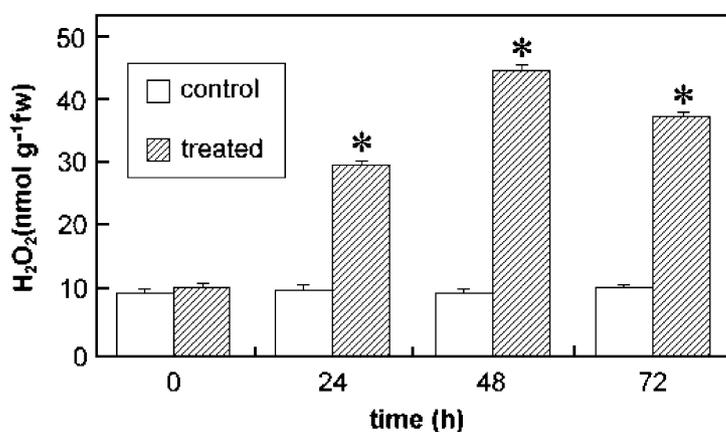
Figure 2. Hydrogen peroxide content of the roots of 5-day-old pumpkin seedlings treated (closed bars) with 50 μM aluminium sulphate. Open bars represent controls. Values represent mean $\pm$ SE of three experiments. * indicates the values that are significantly different from controls (Student's test with $p < 0.01$).

reached a maximum within 48 h (Table 1), it can be suggested that Al treatment induced an oxidative burst, at least transiently. The increased H_2O_2 production in treated roots was also accompanied by an increase in APX activity during the treatment period (Fig. 3A). Although APX activity also increased in control roots, the increase was markedly enhanced in treated roots (approximately 80% at 48 h). The level of AsA was also higher in treated than in control roots (Table 2), suggesting that pumpkin seedlings reacted to Al-induced production of H_2O_2 by expressing higher levels of such scavenging system as the AsA–APX system. Moreover, not only AsA and APX were involved, but other components of the AsA–glutathione system were also involved. In fact, AFRR activity was higher in treated than in control roots (Fig. 3B). This increased activity contributed to the reduction to AsA of the ascorbate free radical (AFR) formed upon the APX-catalysed oxidation of AsA by H_2O_2. Dose–response correlation confirmed that the changes in APX and AFRR activities were induced by Al treatment (Fig. 4). Neither DHAR nor GR

activities changed significantly in treated roots (Figs. 3C and D). However, also these enzymes normally contribute to maintaining the AsA system in the reduced state. The fact is that the redox state of AsA proved unaffected by the Al treatment (Table 2).

Discussion

The toxicity of aluminium was manifested in dark-grown pumpkin seedlings by the rapid inhibition of root growth. No apparent change in shoot growth was observed. Maximum accumulation of aluminium in the root tip was correlated with increased production of H_2O_2, indicating that an oxidative burst was involved in the toxicity of Al, at least in the short term. The level of H_2O_2 reached a maximum within 48 h, and then tended to decline even if it was still much higher than that of the control. The decline in the H_2O_2 level could be due to the scavenging action of the AsA–APX system
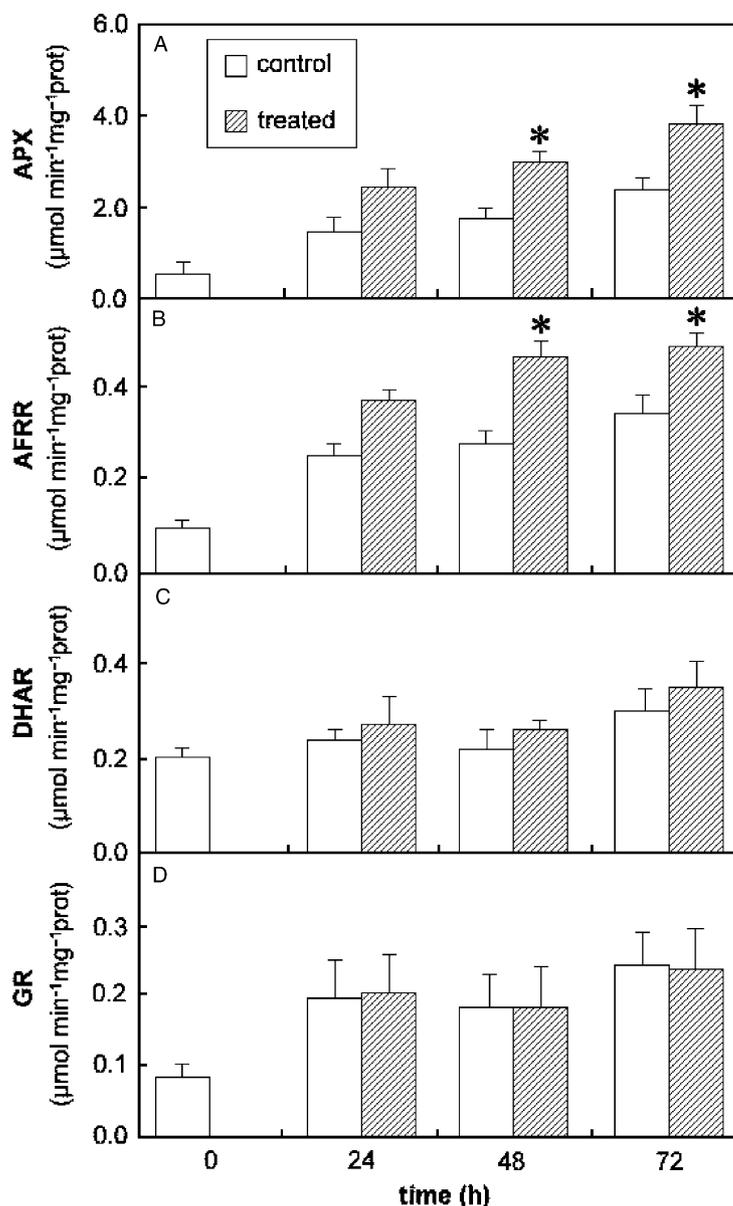
Figure 3. Ascorbate peroxidase (A), ascorbate free radical reductase (B), dehydroascorbate reductase (C), and glutathione reductase (D) specific activities of the roots of 5-day-old pumpkin seedlings treated (closed bars) with $50\,\mu M$ aluminium sulphate. Open bars represent controls. Values represent mean $\pm$ SE of three experiments. * indicates the values that are significantly different from controls (Student's test with $p < 0.05$).

Table 2. Effect of $50\,\mu M$ aluminium sulphate on the AsA and DHA content of pumpkin roots

Treatment duration (h)	C AsA	T AsA	C AsA+DHA	T AsA+DHA
24	870 ± 80	1075 ± 90	912 ± 85	1138 ± 100
48	1109 ± 100	1420 ± 130	1178 ± 120	1495 ± 150
72	1407 ± 140	1707 ± 150	1484 ± 150	1806 ± 180

Five-day-old seedlings were bathed for up to 72 h with $50\,\mu M$ $Al_2(SO_4)_3$ in $100\,\mu M$ $CaCl_2$ and 300 mg samples of root tips, 5 mm long, were analyzed for AsA and DHA contents at the indicated times (C=control, T=treated).

since we found both increased APX activity and an increased level of AsA in treated plants. The increases of both APX and AsA during germination should be considered normal events, as they were also shown for the germination of wheat (De Gara et al., 1997). Actually, we also found both APX and
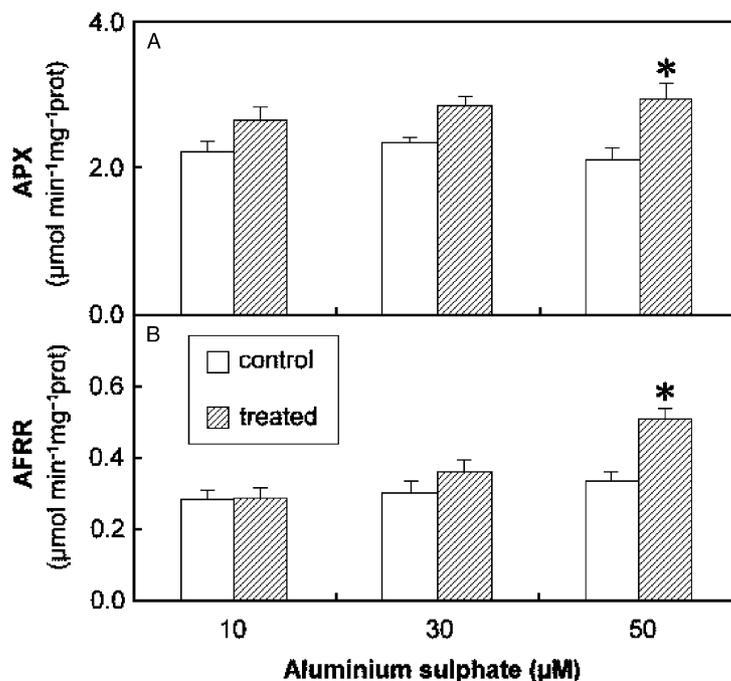
Figure 4. Dose–response relationship for the APX (A) and AFRR (B) specific activities of control (open bars) and treated (closed bars) roots of 5-day-old pumpkin seedlings. Values represent mean $\pm$ SE of three experiments. * indicates the values that are significantly different from controls (Student's test with $p < 0.05$).

AsA to increase in control plants, but in treated plants the increase was significantly higher. This greater increase was undoubtedly a response to the increased H_2O_2 level. However, the amount of Al accumulated inside the root was notably reduced at times longer than 48 h, while the uptake of calcium increased at the same time. Therefore, the decline of the H_2O_2 level might not only be due to the scavenging action of the AsA–APX system but also to the decreased accumulation of Al.

The reason for the decreased amount of Al found in roots at times longer than 48 h is now under investigation. One reason could be the transport of Al to the shoot. In fact, contrary to some reports in the literature, we found higher levels of Al in the hypocotyls of treated seedlings than in controls (data not shown). However, the transport to the shoots should only play a minor role. A major role could be played by the export of Al from the roots into the soil, as well as the exclusion of Al from uptake into the roots. It has been reported that one specific response to Al stress in Al tolerant plants is the secretion of malate or citrate, which would sequestrate Al into the apoplast (Ryan et al., 1995, Yang et al., 2000). Although pumpkin has proved not to be an Al tolerant plant, some similar mechanisms could also act in pumpkin roots.

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