PERGAMON

Phytochemistry 62 (2003) 181–189

PHYTOCHEMISTRY

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Aluminum-induced oxidative stress in maize

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Received 29 March 2002; received in revised form 27 September 2002

Abstract

The relation between Al-toxicity and oxidative stress was studied for two inbred lines of maize (*Zea mays* L.), Cat100-6 (Al-tolerant) and S1587-17 (Al-sensitive). Peroxidase (PX), catalase (CAT) and superoxide dismutase (SOD) activities were determined in root tips of both lines, exposed to different Al^{3+} concentrations and times of exposure. No increases were observed in CAT activities in either line, although SOD and PX were found to be 1.7 and 2.0 times greater than initial levels, respectively, in sensitive maize treated with 36 μM of Al^{3+} for 48 h. The results indicate that Al^{3+} induces the dose- and time dependent formation of reactive oxygen species (ROS) and subsequent protein oxidation in S1587-17, although not in Cat100-6. After exposure to 36 μM of Al^{3+} for 48 h, the formation of 20 ± 2 nmol of carbonyls per mg of protein was observed in S1587-17. The onset of protein oxidation took place after the drop of the relative root growth observed in the sensitive line, indicating that oxidative stress is not the primary cause of root growth inhibition. The presence of Al^{3+} did not induce lipid peroxidation in either lines, contrasting with the observations in other species. These results, in conjunction with the data presented in the literature, indicate that oxidative stress caused by Al may harm several components of the cell, depending on the plant species. Moreover, Al^{3+} treatment and oxidative stress in the sensitive maize line induced cell death in root tip cells, an event revealed by the high chromatin fragmentation detected by TUNEL analysis.

Keywords: Aluminum toxicity; Oxidative stress; Protein oxidation; Lipid Peroxidation; Maize; ROS; Cell death

1. Introduction

Various kinds of environmental stress induce the formation of reactive oxygen species (ROS) in plant cells (for a review, see Hippeli et al., 1999; Breusegem et al., 2001). The sources of environmental stress include changes in temperature, mechanical shock, UV light, exposure to ozone, water deficiency, and an excess of metallic ions. Under normal physiological conditions, cells produce ROS by means of the reduction of molecular oxygen (Hippeli et al., 1999), but under conditions of environmental stress this production is increased. All cells possess a defensive system, consisting of various enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (PX) and reductase. These enzymes efficiently reduce ROS under normal circumstances, but if complete reduction does not occur, as under conditions of increased production, the result may be a state of oxidative stress leading to the oxidation of biomolecules (lipids, proteins, and DNA, for example) (Richter and Schweitzer, 1997) or even cell death (Buckner et al., 2000; Jones, 2000).

The toxicity caused by aluminum in plants in acid soils is a well known example of such environmental stress (for a review, see Kochian, 1995; Ma, 2000; Matsumoto, 2000; Ryan and Delhaize, 2001; Ma et al., 2001). The primary effect of Al toxicity is the inhibition of root elongation; however, the molecular mechanisms involved in this toxicity are unknown (Matsumoto, 2000). Unanswered questions include whether or not this toxicity is due to interactions of the ion at specific sites, which may be located in the cell walls (interactions

Abbreviations: CAT: catalase; 2; 4-DNPH: dinitrophenylhydrazine; MDA: malondialdehyde; NBT: p-nitro blue tetrazolium chloride; PX: peroxidase; ROS: reactive oxygen species; RRG: relative root growth; SOD: superoxide dismutase; TBA: Thiobarbituric acid; TEP: tetraethoxypropane; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling

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PII: S0031-9422(02)00491-0

with pectins and hemicelluloses; Horst, 1995), in the symplasm (inhibition of cell division and the binding of Al to the DNA; Clarkson, 1995; Liu and Jiang, 1991), in the plasma membrane (interaction with lipids; Dellers et al., 1986; Jones and Kochian, 1997) or in the formation of callose (Wissemeir et al., 1992).

It has been suggested that Al^{3+}, the most toxic of the soluble forms of Al (Parker et al., 1988) induces oxidative stress, since this ion is involved in various processes, including an increase in enzyme activity (SOD and PX) related to ROS and lipid peroxidation in soybeans (*Glycine max)* (Cakmak and Horst, 1991), peas (*Pisum sativum)* (Yamamoto et al., 2001) and tobacco plants (*Nicotiana tabacum* L.) (Ono et al., 1995; Yamamoto et al., 1997; Ikegawa et al., 2000), as well as changes in the expression of various genes induced by Al in Arabidopsis (Sugimoto and Sakamoto, 1997; Richards et al., 1998), tobacco (Ezaki et al., 2000) and wheat (*Triticum aestivum* L.) (Snowden and Gardner, 1993; Cruz-Ortega et al., 1997; Hamel et al., 1998). The oxidative stress provoked by Al^{3+} can also induce cell death, as has been observed in the root tips of barley (Pan et al., 2001), and wheat (Deslile et al., 2001). It has been observed that addition of Al^{3+} causes an immediate increase in the quantity of this ion in tobacco cells, although this does not significantly increase the uptake of Evans blue nor the peroxidation of lipids, which suggests that the Al^{3+} has not harmed the membrane. However, the addition of Fe^{2+} to cells exposed to Al^{3+} for 12 h caused immediate lipid peroxidation and the uptake of Evans blue several hours later, suggesting that under these conditions the accumulation of Al in the tobacco cells modified the physical structure of the membrane, thus facilitating the propagation of lipid peroxidation, initiated by Fe^{2+}, and eventually causing the death of the cell (Ono et al., 1995; Yamamoto et al., 1997; Ikegawa et al., 2000). Recently, Yamamoto et al. (2001) have suggested that peroxidation of lipids stimulated by Al^{3+} in peas is a relatively early symptom, but not the direct cause of the growth inhibition of the root by the ion.

All the results reported in the literature associate Al-induced oxidative stress with lipid peroxidation in plants, suggesting, as proposed by Yamamoto et al. (2001), that this process in biological membranes is the most prominent symptom of oxidative stress in plants and animals. No information is available relating the induction of ROS by Al^{3+} to oxidative stress and degradation of ROS by other processes, such as protein oxidation in plants. The goal of the present paper was thus to investigate the relation between these three processes (ROS, oxidative stress and protein oxidation) using two inbred lines of maize (*Zea mays* L.), Cat100-6 (Al- tolerant) and S1587-17 (Al-sensitive). Moreover, the role of Al^{3+} and lipid peroxidation in the induction of cell death in maize will be investigated, to determine whether or not lipid peroxidation is a direct cause of this death.

2. Results and discussion

2.1. Effect of Al on relative root growth, SOD, PX and CAT activities

Fig. 1 shows the SOD and PX activities of maize root seedlings of tolerant and sensitive lines treated for 48 h in a nutrient solution with various concentrations of $AlCl_3$ at pH 4.1. The relative root growth (RRG, Fig. 1A) of the two lines is dependent on Al^{3+} concentration, with the presence of more than 12 μM affecting the S1587-17, whereas Cat100-6 was affected only at higher concentrations. At higher Al concentrations, although both lines had reduced RRG, Cat100-6 was less affected than S1587-17. Organic acid anion exudation has been described as an important mechanism for the exclusion of Al^{3+} from root tip cells by tolerant

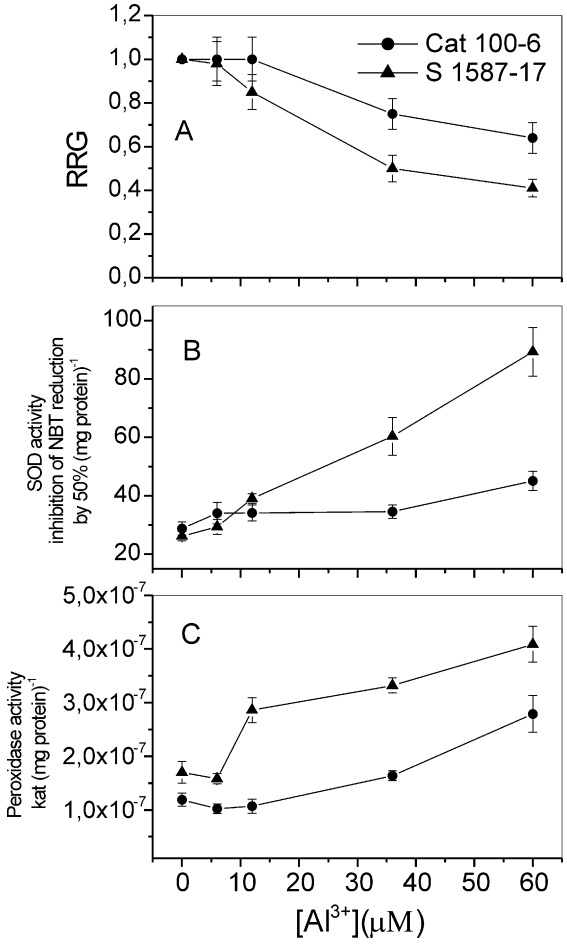

Fig. 1. Dose–response curves for inhibition of relative root growth (RRG), superoxide dismutase (SOD) and peroxidase activity after 48 h exposure of Cat100-6 and S1587-17 exposure in nutrient solutions containing Al^{3+}. The data are the mean ± S.D. ($n = 10$ for RRG and $n = 4$ for enzyme activities).

plants (Myasaka et al., 1991; Delhaize et al., 1993; Pellet et al., 1995; Zheng et al., 1998; Ma, 2000; Yang et al., 2000). Jorge et al. (2001) have shown that Cat100-6 exudes organic acid anions, mainly citrate, as a defense mechanism, and this can be used to exclude Al from the root tips. The sensitive line also exudes citrate, but 3.5 times less than the tolerant line exposed to 36 μM Al^{3+}.

SOD activity is responsible for the degradation of superoxide radicals ($O_2^{\bullet -}$), to produce O_2 and H_2O_2. This activity (Fig. 1B) in the S1587-17 root apex increased almost linearly with an increase in Al^{3+} concentration, while that in the Cat100-6 remained practically constant; the Cat100-6 with 60 μM Al^{3+} had the same SOD activity as the S1587-17 with 12 μM Al^{3+}. The increase in SOD activity of the S1587-17 line indicates that exposure to Al^{3+} induces the formation of superoxide radicals in a quantity greater than that which the pre-existent SOD can remove, a result which suggests that Al^{3+} induces the cell to initiate SOD synthesis. Similar dose-dependent results were found in soybean (Cakmak and Horst, 1991), although the study was conducted only with a sensitive cultivar. In the present paper, analyses were made for both tolerant and sensitive lines with similar genetic backgrounds, facilitating the comparison of results and the evaluation of whether the changes observed are due to effects of Al^{3+} toxicity in general or to a genotype-specific toxic reaction.

PX activity is responsible for the degradation of hydrogen peroxide. The sensitive maize line showed greater PX activity than the tolerant one after 48 h at 6,12, 36 and 60 μM of Al^{3+} treatments. With 60 μM of Al^{3+}, the Cat100-6 showed PX activity equivalent to that found for the S1587-17 treated with 12 μM. The RRG after 48 h tends to be inversely proportional to SOD and PX activity (Fig. 1), which suggests that the increase in synthesis of superoxide radicals and the formation of H_2O_2 may be related to Al toxicity.

Fig. 2 relates the RRG, SOD and PX parameters with time. At 12 μM, the RRG of the primary root of sensitive plants decreased after 12 h and remained unchanged at latter times (Fig. 2A). At higher Al concentrations a continuous decrease of RRG was observed. After prolonged incubation in these highly toxic Al concentrations, the termination of all root growth were observed (results not shown). In tolerant plants the RRG remained constant or increased after 12 h (Fig. 2B). SOD and PX activities had little changes at 24 h in both lines in response to 36 μM of Al. However, after 24 h in the presence of Al^{3+} both activities increased drastically for the S1587-17 (Fig. 2C and D). In the Al-tolerant Cat100-6, a slight increase in both SOD an PX activities was also observed, but this increase was significantly lower than those observed for S1587-17.

These results indicate that a transient block in root elongation, such as that observed when the sensitive and tolerant lines were exposed to 12 and 36 μM, respectively, is not caused by oxidative stress. Rather, our data suggest that the oxidative stress takes place only after a sustained inhibition of root growth. Consistent with this evidence, Yamamoto et al. (2001) showed that butylated hydroxyanisole (BHA), a lipophilic antioxidant, decreases lipid peroxidation in pea roots, but does not prevent root growth inhibition due to Al. Thus, the data show that the inhibitory effect of Al^{3+} on the root cells is taking place earlier than the activation of some plant defenses against oxidative stress, reinforcing the hypothesis that oxidative stress is not the primary cause of root growth inhibition. The data from protein oxidation assays agree with this hypothesis (see below).

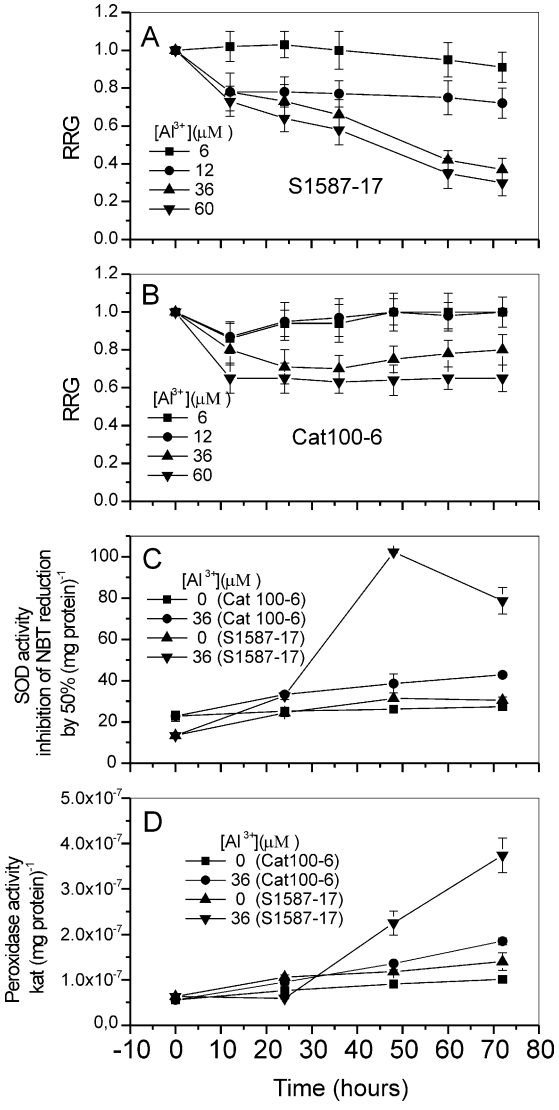

Fig. 2. Time-course of Al^{3+} inhibition of relative root growth, superoxide dismutase (SOD) and peroxidase activity of Cat100-6 and S1587-17 exposed to 36 μM $AlCl_3$ in nutrient solution. The data are the mean ± S.D. ($n = 10$ for RRG and $n = 4$ for enzymes activities).

It is worth noting that the observed balance between PX and SOD activities is very important in the antioxidant defense system of the cell (Gille and Sigler, 1995) because the degradation of superoxide radicals by SOD produces H_2O_2, which would lead to oxidative stress without a parallel increase in the PX activity in the cell (Gosset et al., 1996; Cakmak and Horst, 1991; Gonzáles et al., 1998). Surprisingly, in contrast to the increased levels of PX, CAT activities were not detected in the root tips (results not shown). However, similar results have already been observed in coffee seeds exposed to oxidative stress caused by low temperatures (Queiroz et al., 1998). The results obtained with these maize root seedlings show that H_2O_2 degradation occurred due to activation of peroxidases rather than catalase.

2.2. Lipid peroxidation and protein oxidation

Lipid peroxidation in response to Al has been reported in several species: Yamamoto et al. (2001) showed the induction of lipid peroxidation in pea plants after 4 h of exposure to Al^{3+}; Cakmak and Horst (1991) also observed an increase in lipid peroxidation of a sensitive soybean cultivar after 24 h of treatment; Basu et al. (2001) found that the levels of malondialdehyde (MDA) are directly related to the inhibition of root growth in *Brassica napus*.

The lipid peroxidation of the maize lines was thus investigated after treatment with 36 μM Al^{3+} for 48 h. Once no such peroxidation was determined by the thiobarbituric acid (TBA) method for either of the lines (Fig. 3A), two other methods were also applied for its determination: xylenol orange assay and HPLC. The quantity of hydroperoxides detected by the xylenol orange assay was statistically the same for the tolerant and sensitive lines (Fig. 3B), and when the samples obtained from the acidic hydrolysis of tetraethoxypropane (TEP) was injected into a HPLC system, both lines revealed a retention time of 2.48 min and the same area. The retention time of the MDA standard was 2.36 min, exactly the same found when a known quantity of standard was injected with a control sample, indicating that the signal at 2.48 min is not MDA (results not shown).

Thus, unlike the results obtained with peas (Yamamoto et al., 2001) and soybeans (Cakmak and Horst, 1991), our results show that in maize, lipid peroxidation is not induced by Al^{3+} treatment, indicating that lipids are not the primary cellular target of oxidative stress. Therefore the target of oxidative stress varies depending on the plant species.

Since the excess ROS induced by the presence of Al^{3+} did not cause lipid peroxidation, the putative oxidation of proteins was investigated in these maize lines. The difference in carbonyl content between the controls and the Al-treated plants was used to determine the amount of protein oxidation occurring in root tips. The quantity of carbonyls in Al-sensitive plants increased with an increase in Al^{3+} concentration, although no changes were observed in the tolerant line (Fig. 3C). An increase in carbonyls was detected after 24 h of exposure to Al^{3+} in the sensitive line, reaching a peak of nearly 82 nmol mg^{-1} of proteins after 48 h, although the level in the tolerant line continued basically unchanged (Fig. 3D). To our knowledge, this is the first report on protein oxidation rather than lipid peroxidation due to Al toxicity in plants. In agreement with the hypothesis that oxidative stress is not the primary cause of root growth inhibition, we observed that while the RRG drops after

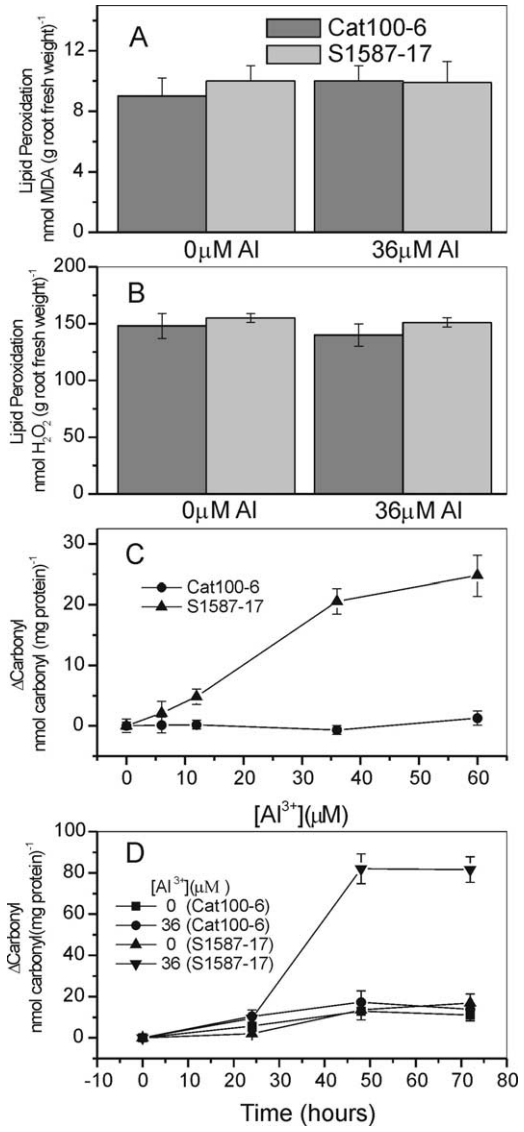

Fig. 3. Effect of Al^{3+} on lipid peroxidation and protein oxidation in Cat100-6 and S1587-17. Lipid peroxidation (A and B) or protein oxidation (C) was measured after 48 h incubation with various concentrations of Al^{3+}. Protein oxidation was measured over time (D) for fixed concentrations of Al^{3+}, as indicated in the figure. The data are the mean ± S.D. ($n = 2$ for lipid peroxidation and $n = 4$ for protein oxidation).

12 h at 36 μM Al in the sensitive line (Fig. 2A), protein oxidation at 24 h was near the basal levels (see below, Fig. 3D).

The mechanism for the production of ROS in plants in response to stress and by cellular respiration is as yet unknown. It seems that $O_2^{\bullet -}$ production can be catalyzed by various enzymes, such as the peroxidases (Auh and Murphy, 1995) and NADPH oxidase (Bolwell et al., 1998) which may be regulated by Rac-related GTP-binding proteins (Jumok et al., 2000). One possible mechanism is presented in Eqs. (1)–(3), which suggests that the initial formation of $O_2^{\bullet -}$ is catalyzed by NADPH (Hancock et al., 2001); this is then degradated into H_2O_2 and O_2, with the former being catalyzed by Fe^{2+} into the hydroxyl radical (Hippeli et al., 1999).

$$2O_2 + NADPH = 2O_2^{\bullet -} + NADP^+ + H^+ \quad (1)$$

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \overset{SOD}{=} H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 = Fe^{3+} + OH^{\bullet} + OH^- \quad (3)$$

Halliwell and Gutteridge (1999, p. 315) suggested that the oxidation of proteins to form carbonyls occurs via the $OH^{\bullet}$ radical, since neither H_2O_2 nor $O_2^{\bullet -}$ are reactive enough to provoke oxidation. The formation of carbonyls is a process which involves a site-specific mechanism in proteins (Stadtman and Oliver, 1991; Stadtman, 1992; Becana et al., 1998). In the model proposed by Stadtman and Oliver (1991; Stadtman, 1992), Fe^{2+} binds to this specific binding site of proteins, with the protein–Fe(II) complex then reacting with H_2O_2 to generate in situ ROS [hydroxyl and ferryl, $(FeO)^{2+}$] radicals. These radicals will not be liberated to the surroundings, although they may react with amino acid side chains of the metal–ligand site, thus forming an alkyl radical; the terminal carbon is then converted into a carbonyl.

The protein oxidation in both S1587-17 and Cat100-6 probably involves a site-specific mechanism, as proposed by Stadtman and Oliver (1991; Stadtman, 1992). Our evidence indicates that the differences in protein oxidation in the two maize lines are related to the levels of antioxidant defense. Both SOD and PX activities are 3-fold higher in the sensitive maize line after 48 h at 36 μM Al (Fig. 1B and C), however the protein carbonyl levels are about eight times higher than in the tolerant maize line (Fig. 3D). These results suggest that Al^{3+} induced the production of superoxide radicals and, as a consequence, SOD reaction, as well as the increase in hydrogen peroxide and hydroxyl ($OH^{\bullet}$) radicals in the cell. The accumulation of carbonyls in the sensitive maize line thus indicates that the quantity of radicals generated exceeded the capacity of the antioxidant defensive system, whereas in the Al-tolerant maize ROS was eliminated by plant defenses. Certainly one such defense is the exudation of citrate, an Al-chelating compound, which is 3.4-fold higher in Cat100-6 (Jorge et al., 2001). Upon citrate exudation, a lower amount of Al^{3+} would be left to disturb the cellular homeostasis and cause the oxidative stress in the tolerant line.

On the other hand, the peroxidation of lipids probably also starts with the hydroxyl radical (Halliwell and Gutteridge, 1999, p. 296). These authors have pointed out that scavengers of $OH^{\bullet}$ do not inhibit the process, which suggests that the Fe^{2+} bound to the membrane and exposed to the attack of H_2O_2 generates $OH^{\bullet}$ locally. In this sense, the $OH^{\bullet}$ formed will react locally and immediately with the lipids in the membrane.

The absence of lipid peroxidation in maize is quite intriguing. A possible explanation would be that the Fe^{+2} bound to the membrane is not exposed to the attack of H_2O_2, while in other plants such as peas (Yamamoto et al., 2001) and soybeans (Cakmak and Horst, 1991) these sites would be exposed for the catalysis of $OH^{\bullet}$ formation. In neither maize (data not shown) nor peas (Yamamoto et al., 2001) does the addition of Fe^{2+} to the nutrient solution have any effect on lipid peroxidation in the presence of Al^{3+}. This can be explained by the hypothesis that when exposed sites are absent the Fe^{2+} has no way of catalyzing the reaction, whereas when the exposed sites are present but already saturated (peas) the reaction continues normally. The explanation of this difference between species necessitates further investigation.

2.3. Al treatment induces cell death

Some authors have suggested that oxidative stress may induce plant cell death by the generation of ROS (Buckner et al., 2000; Breusegem et al., 2001). We used the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) reaction to detect cell death in the root tips from both maize lines in response to a 48 h Al exposure. A large difference between images obtained from Cat100-6 and those from S1587-17 was observed. In tolerant maize (Fig. 4A), almost all the nuclei were green, while in sensitive maize (Fig. 4B) almost all were brown, indicating high chromatin fragmentation and, consequently, cellular death. The relative increase in cell death was further pointed out by Evans blue uptake, using a spectrophotometric assay according to Baker and Mock (1994). Evans blue uptake was 3.4 times higher in sensitive root tips after 48 h exposed to 36 μM Al (results not shown). Whether this is due to necrosis or programmed cell death remains to be determined, since necrotic cells may also give a positive signal in TUNEL reactions (see Danon et al., 2000, and references therein). It is tempting to speculate that in the soil, when the root reaches a region containing highly toxic levels of Al, a mechanism could be activated

to destroy the root tip cells, to break the apical dominance and thus induce the formation of secondary roots to explore other soil portions with lower levels of Al. Pan et al. (2001) found evidence that the inhibition of root elongation in barley (*Hordeum vulgare*) after 8 h of Al treatment is mainly caused by Al-induced programmed cell death, although more investigation should be made to prove this hypothesis. Early work from Matsumoto's group showed that Al^{3+} binds to DNA, causing condensation and stabilization of the chromatin, decreasing the rate of RNA synthesis, which consequently would inhibit cellular functions (Matsumoto and Morimura, 1980; Matsumoto, 1988). Recently, Mouzannar et al. (2001) found that hydrogen peroxide causes a rapid and extensive chromatin fragmentation in oligodendrocytes. These findings, together with the higher levels of SOD and PX activities observed in the sensitive line, indicate that the production of H_2O_2 and the block of cellular functions (such as DNA repair mechanisms) may explain the chromatin fragmentation detected in response to Al.

Ikegawa et al. (2000) suggested that the Al-enhanced peroxidation of lipids is a direct cause of cell death in tobacco cells. The present results have shown that lipid peroxidation is not essential for cell death in maize and suggest that oxidative stress can induce cell injury by several pathways, as pointed out for mammalian cells by Halliwell and Gutteridge (1999, p. 341).

In summary, increased PX and SOD activities in the sensitive line induced by Al^{3+} treatment suggest that this ion induces ROS formation, leading to protein oxidation, a clear indication that oxidative stress is taking place. However, oxidative stress is not a primary cause of root growth inhibition. Although lipid peroxidation, observed in other species, was not found in maize, we detected protein oxidation and cell death in response to Al, indicating that the several known pathways affected by oxidative stress in mammals may be activated in a plant species-dependent way. Further investigation on other plant species and also other possible manifestations of oxidative stress will shed light on this issue.

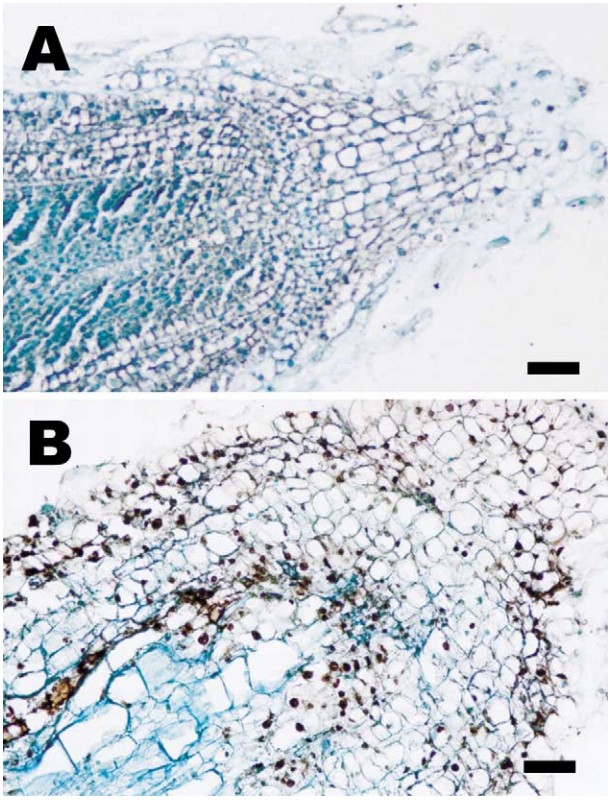

Fig. 4. Detection of chromatin fragmentation in root sections of Al-tolerant Cat100-6 (A) and Al-sensitive S1587-17 (B) maize lines grown in nutrient solution at pH 4.1 containing 36 μM of Al^{3+}. The dark nuclei indicated high levels of chromatin fragmentation in S1587-17 root. Swelling of the Al-sensitive maize roots has occurred as evidenced by the larger root of tip cells portrayed. Bar = 100 μm.

3. Experimental

3.1. Plant material, seedling growth and Al-treatment

Inbred lines of Al-tolerant Cat100-6 maize (*Zea mays* L.) and its somaclonal variant S1587-17 (Al-sensitive) (Moon et al., 1997) were obtained from the collection of Germplasm Bank of the Universidade Estadual de Campinas, Campinas, Brazil. The plants were grown in the field and self-pollinated.

Seedling growth in a nutrient solution and RRG calculations were carried out according to Jorge et al. (2001). Since the toxicity of Al is related to the activity of free Al^{3+} rather than to the molar concentration, the activity of this ion at pH 4.1 (Table 1) was calculated using Geochem 2.0 software (Parker et al., 1995). The plants were transferred to a nutrient solution (Jorge et al., 2001) in the absence (control) and presence of 6, 12, 36 and 60 μmol l^{-1} of $AlCl_3$. The $AlCl_3$ solution was freshly prepared and standardized according to Kinnunem and Merikanto (1955). The solutions were continuously aerated and the plants exposed at 26 ± 1 °C under a photoperiod of 16 h at 16 W m^{-2}.

Table 1
Total concentration and free activity (a non-dimensional quantity) of Al^{3+} in nutrient solutions, at pH 4.1, used in treatment of plants to relative root growth (RRG), enzyme activities, protein oxidation and lipid peroxidation[a]

$[Al^{3+}]$ (μmol l^{-1})	Free Al^{3+} activity ($\times 10^6$)
6	2.7
12	5.4
36	16.2
60	27.0

[a] For the calculation of the Al^{3+} activity, 1 mol dm^{-3} of solution was used as standard.

3.2. Enzyme activities, protein oxidation, lipid peroxidation and cell death

After treatment in the nutrient solution with and without Al^{3+}, the plants of the two maize lines were washed three times with deionized distilled water, and the root tips were then excised (5–6 mm) and used for the various conducted experiments.

3.2.1. Enzyme activities

Enzyme activities in each extract were determined spectrophotometrically using a diode array spectrophotometer (8452 A, Hewlett Packard, USA). These assays were conducted in a total volume of 2 ml at 25 °C and the results were the mean of four independent experiments using 15–20 root tips. Root tips were homogenized in 1 ml of HEPES-KOH buffer (50 mM, pH 7.8) containing 0.1 mM EDTA and total SOD (EC 1.15.1.1) activity was determined by inhibition of *p*-nitro blue tetrazolium chloride (NBT) photoreduction (Giannopolitis and Ries, 1977). A fluorescent lamp (Sylvannia F15WT2) was positioned 4 cm from the samples for 5 min. One unit of SOD is the amount of enzyme that inhibits NBT photoreduction by 50%, monitored at 560 nm. To measure CAT and PX activities, root tips were homogenized in 1 ml of phosphate buffer (50 mM, pH 6.8). CAT (EC 1.11.1.6) and PX (EC 1.11.1.7) were assayed as outlined by Cakmak and Horst (1991). For CAT, the decrease in absorbance at 240 nm due to degradation of H_2O_2 ($\varepsilon = 39{,}4$ l mmol^{-1} cm^{-1}) was monitored. For PX, the reaction mixture contained 50 mM phosphate buffer (pH 6.8), 8 mM guaiacol, 8 mM H_2O_2 and protein extract. The increase in absorbance due to tetraguaiacol formation was recorded at 470 nm ($\varepsilon = 26.6$ l mmol^{-1} cm^{-1}). Proteins in each extract were assayed according to the method of Bradford (1976).

3.2.2. Protein oxidation

The reaction of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) was used to determine the amount of protein oxidation, as described in Levine et al. (1990). Protein extract was obtained by homogenization of 15–20 root tips in a phosphate buffer (25 mM, pH 7.0). After DNPH-reaction, the carbonyl content was calculated by absorbance at 374 nm, using the extinction coefficient for aliphatic hydrazones (22 l mmol^{-1} cm^{-1}) and expressed as nmol carbonyl/mg protein.

3.2.3. Lipid peroxidation

The level of lipid peroxidation in root-tip segments was determined by three different methods: TBA, HPLC and xylenol orange. The TBA method (Baccouch et al., 1998) determines MDA as an end product of lipid peroxidation by reaction with TBA. After excision, the root tips were blotted dry and immediately weighed (100 mg) and homogenized in 1 ml of reaction mixture. Absorbance at 532 nm was determined and the extinction coefficient of 155 l mmol^{-1} cm^{-1} was used to calculate the concentration of MDA. An analytical curve was obtained using tetraethoxypropane (TEP) in an acidic solution to generate MDA.

The amount of MDA in the samples was also determined using HPLC (Bull, 1985). Around 30 root tips were homogenized in a phosphate buffer (50 mM, pH 6.8) and after centrifugation, samples were filtered through 0.45 μm disposable filter (Sigma) and injected into a HP ODS Hypersil 5 μm column (4.6×100 mm) in a HPLC (Waters 600E). Samples were eluted isocratically at 1.0 ml min^{-1} with acetonitrile [14% (v/v)] in tetrabutylammonium bromide (50 mM) in phosphate buffer (1.0 mM, at pH 6.8). Detection was based on absorbance at 266 nm using a tunable absorbance detector (Waters 484) and a data module integrator (Waters 746). Retention time and the relation between MDA concentration and area were obtained using TEP in an acidic solution.

Lipid peroxidation was also determined based on the formation of an Fe(III) xylenol orange complex, measured at 580 nm (Hermes-Lima, 1995). After excision, root tips were blotted, immediately weighed (100 mg) and protein extract was obtained by homogenization of root tips in phosphate buffer (50 mM; pH 7) and, after centrifugation, 100 μl of this extract was added to the reaction mixture (250 μl of 1.0 mM $FeSO_4$; 100 μl of 0.25 mM H_2SO_4; 100 μl of 1 mM xylenol orange). After 12 h of reaction, the absorbance at 580 nm was measured and reaction termination was checked after the addition of 5 nmol of H_2O_2 and after 1 h incubation.

3.3. Evaluation of cell death

3.3.1. Tunel

Root tip cells exposed to 36 μM of Al^{3+} for 48 h were fixed for 24 h in 3.7% paraformaldehyde (pH 7.4), dehydrated in ethanol and embedded in Paraplast Plus (Oxford, USA). The material was cut into 7-μm-thick sections, rehydrated, and submitted to a TUNEL reaction for the detection of chromatin fragmentation. TUNEL reactions were performed using an *In Situ Cell Death Detection* kit (Boehringer Mannheim, Germany), following the manufacturer's suggestions. Nuclei with high chromatin fragmentation acquired a brownish color, while nuclei with little fragmentation acquired a greenish color. Pictures were obtained using a Zeiss microscope.

3.3.2. Evans blue uptake

After aluminum treatment, seedlings from the two maize lines were washed three times with deionized distilled water and stained for 15 min with an Evans blue aqueous solution (0.25%, w/v). Seedlings were washed

three times with deionized distilled water and the root tips were then excised (5–6 mm). Cell death was evaluated by Evans blue staining as described by Delisle et al. (2001).

Acknowledgements

We would like to thank Dr. Pedro Henrique P. A. Schildknecht of the Instituto de Biologia of UNICAMP for help in cell death detection. PRSB was recipient of a fellowship from FAPESP (project 99/04237-6). This work was supported by grants from the Fundo de Apoio ao Ensino e Pesquisa of UNICAMP (FAEP, project 519292), the European Commission (project INCO II RDT ICA4-CT-2000-30017) and PADCT/CNPq (project 62.0472/98.7).

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