The Role of Lipid Peroxidation in Aluminium Toxicity in Soybean Cell Suspension Cultures

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Z. Naturforsch. **55c,** 957–964 (2000); received July 20/August 29, 2000

Lipid Peroxidation, Aluminium Toxicity, *Glycine max*

The primary reactions leading to Al toxicity in plant cells have not yet been elucidated. We used soybean (*Glycine max* [L.] Merr.) cell suspension cultures to address the question whether lipid peroxidation plays an important role in Al toxicity. Upon transfer to an Al-containing culture medium with a calculated Al^{3+} activity of 15 μM soybean cells showed a distinct and longtime increase in lipid peroxidation within 4h. At the same time a drastic loss of cell viability was observed. Butylated hydroxyanisole (BHA) and *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), two lipophilic antioxidants, were able to almost completely suppress lipid peroxidation in Al-treated cells at a concentration of 20 μM. This effect was dose-dependent for DPPD and was observed at minimum concentrations of 1–2 μM. When lipid peroxidation was suppressed by DPPD or BHA cell viability remained high even in the presence of toxic Al concentrations. These results suggest that Al-induced enhancement of lipid peroxidation is a decisive factor for Al toxicity in suspension cultured soybean cells.

Introduction

Aluminium (Al) is the most abundant metal element in the earth crust. Al-silicates and Al-oxides are important components of many soils around the world. Despite this ubiquity Al does not exert a known function in plant metabolism and most plants try to exclude Al from the cells (Bennet and Breen, 1991). At neutral pH the solubility of soil bound Al is low. Only under acidic conditions higher concentrations of the phytotoxic Al^{3+} in soil solution arise which limit plant growth. Al toxicity is regarded as the major factor of the infertility of acidic soils which occupy approximately 30% of the world's ice free land area (von Uexküll and Mutert, 1995). The first visible effect of Al toxicity on plants is an inhibition of root growth. Both cell division and cell elongation at the root tip are sensitive to Al whereas the mature parts of the root remain uneffected (Ryan *et al.*, 1993; Kochian, 1995). Despite intensive research the molecular mechanisms underlying the phytotoxic effects of Al are not well understood. It was shown that Al^{3+} impairs the functions of cell wall (Horst, 1995), cytoskeleton (Grabski and Schindler, 1995) and DNA (Matsumoto, 1991) but there is increasing evidence that the plasma membrane may be the primary site of Al toxicity (Barceló *et al.*, 1996).

Al^{3+} can bind to the outer surface of the plasma membrane where it stimulates Fe^{2+}-induced peroxidation of membrane lipids (Akeson *et al.*, 1989; Oteiza, 1994). This lipid peroxidation is a radical chain reaction that can lead to a loss of membrane integrity and membrane protein function (Halliwell and Gutteridge, 1989). Enhanced lipid peroxidation is not confined to aluminium toxicity but results from many different plant stress situations like dehydration (Navari-Izzo *et al.*, 1997), heavy metal toxicity (Weckx and Clijsters, 1997) or pathogen attack (Rustérucci *et al.*, 1996). From their investigation of Al-treated soybean roots Horst and co-workers concluded that the measured enhancement of lipid peroxidation was a consequence rather than a cause of Al toxicity (Horst *et al.,* 1992). However, they could not detect lipid peroxidation in Al treated cultered soybean cells (Stass and Horst, 1995). On the other hand, aluminium-enhanced lipid peroxidation seems to be

Abbreviations: BHA, butylated hydroxyanisole; DMSO, dimethyl sulfoxide; DPPD, *N,N'*-diphenyl-*p*-phenylenediamine; FDA, fluorescein diacetate; MDA, malondialdehyde; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

0939–5075/2000/1100–0957 $ 06.00

a decisive factor for Al cytotoxicity in tobacco cell cultures (Yamamoto *et al.*, 1997).

In the present study we used soybean cell suspension cultures to further elucidate the role of lipid peroxidation in Al cytoxicity. Our results support the assumption that in short-time experiments Al toxicity is largely mediated by an increase in lipid peroxidation.

Materials and Methods

Cell culture maintenance and media

Cell suspension cultures of soybean (*Glycine max* [L.] Merr.) cultivar Doko RC were maintained in a combined medium of MS mineral salts (Murashige and Skoog, 1962) and B5 organic compounds (Gamborg *et al.*, 1968) with 30 g/l sucrose and pH 5.7 (pH 5.0 after autoclaving) designated as standard medium. Cultures were subcultivated weekly by transferring 2–3 g fr wt of cells to 40 ml of fresh medium in 200 ml Erlenmeyer flasks. The cultures were shaken at 120rpm in the dark at 25 °C.

For investigating Al toxicity effects in soybean suspension cultures the standard medium was modified as follows. The concentrations of KH_2PO_4 and $CaCl_2*2H_2O$ were reduced to 1.36 mg/l and 14.7 mg/l, respectively, and Na_2-EDTA was omitted. $FeSO_4*7H_2O$ was added after autoclaving of the medium from a freshly prepared solution by sterile filtration prior to the adjustment of the medium to pH 4.0. This medium was designated "pH-medium" and was developed according to the findings of Conner and Meredith (1985) on medium requirements for the study of Al toxicity in cell cultures. pH-medium was used as a control to distinguish effects of the mentioned medium modifications from effects specific to toxic Al ions. An aluminium toxic medium was prepared by adding 267 mg/l $Al_2(SO_4)_3*18H_2O$ to the pH-medium from a freshly prepared solution prior to adjusting the medium to pH 4.0. Thus, an Al concentration of 0.8 mM and an Al^{3+} activity of about 15 μM were obtained according to calculation with GEOCHEM-PC (Parker *et al.*, 1995). The aluminium sulfate solution was prepared using destilled water that was preacidified with HCl in order to avoid formation of polynuclear Al species.

Experimental design

On the fourth day after subculture cells were washed in pH-medium and 1 g fr wt of cells was transferred to 40 ml of standard medium, pH-medium or Al toxic medium. At different time intervals lipid peroxidation and cell viability were determined. The lipophilic antioxidants DPPD (*N,N'*-diphenyl-*p*-phenylenediamine) and BHA (butylated hydroxyanisole) were dissolved in DMSO and added to the fresh culture medium prior to the transfer of cells. Final concentration of DMSO in the culture medium was 0.1% (v/v).

Determination of lipid peroxidation

The peroxidation of lipids in cells was assessed by the TBA (thiobarbituric acid) test for MDA (malondialdehyde) according to Ono *et al.* (1995). At given times 10 ml of cell suspensions were withdrawn and centrifuged. Cells were then washed twice with sucrose-free standard medium in order to avoid development of color due to sugar from the medium. The cells were resuspended in 1 ml of 0.1% (w/v) TCA and disrupted using a sonifier (Sonorex RK 100 H, Bandelin; Berlin, Germany) at 5 °C. An aliquot of 0.5 ml of the sonicate was added to 3 ml of 1% H_3PO_4 (v/v) and 1 ml of 0.6% (w/v) TBA. The mixture was placed in a water bath with 95 °C for 45 min and then cooled to room temperature in a water bath at 5 °C. 4 ml of 1-butanol was added with vigorous mixing. The separation of the butanol phase and the aequous phase was facilitated by centrifugation. The absorbance of the TBA-MDA complex in the butanol phase was determined at 532 nm and the non-specific absorption at 520 nm was substracted. The amount of MDA was calculated using the molar extinction coefficient ε=155,000.

Determination of cell viability

The viability of cells was determined using phenosafranine and FDA (fluorescein diacetate) according to Widholm (1972). One drop of cell suspension was mixed with one drop of phenosafranine solution (0.1% w/v in standard medium) on a microscope slide and a coverslip was lowered over the cells. After 5 min the slide was viewed with a microscope and stained (dead) and unstained (viable) cells were counted.

A FDA stock solution was prepared by dissolving 250 mg FDA in 50 ml acetone. This solution was stored in the freezer and was diluted with standard medium to give a 0.01 (w/v) FDA solution. One drop of this solution was mixed with one drop of cell suspension as mentioned above for phenosafranine. After 5 min the slide was viewed with a fluorescence microscope. The number of stained (viable) cells was compared to total cell number as seen under white light.

Results

Lipid peroxidation and viability of cells after transfer to different media

The soybean cell cultures showed stable growth with a weekly subculture and an average yield of 10 g fr wt/40 ml after seven days. The fine cell suspensions consisted of single cells and small cell aggregates. Cells were used for the experiments on day 4 after subculture, because at that time the cultures were in the exponential growth phase. In order to investigate toxic effects of Al in cell cultures it is necessary to partly modify the standard culture media (Conner and Meredith, 1985). Three different culture media were used in our experiments. The standard medium (MS/B5) was modified to a pH-medium (Al free) that would allow the generation of toxic activities of Al^{3+}. The calcium and phosphate contentrations were reduced, EDTA was omitted and the pH value was lowered to 4.0. An Al toxic medium was obtained by adding aluminium sulfate to the pH-medium and adjusting the pH-value to 4.0. Therefore, the pH-medium could be used as a control for experiments on Al toxicity because it showed all the modifications that were necessary to allow toxic activities of Al^{3+} but did not contain aluminium.

Within two hours after transfer of cells to Al toxic medium with an Al concentration of 0.8 mM and an Al^{3+} activity of 15 μM as calculated by GEOCHEM-PC (Parker *et al.*, 1995) the viability of cells dropped to 35% and it further decreased reaching values of under 10% after eight hours (Fig. 1A). After 43 hours hardly any viable cells could be detected. When cells were transferred to pH-medium cell viability also decreased rapidly to about 35% but remained on that level and finally showed a small increase up to 50% viability after 43 hours. Upon transfer of cells to standard medium cell viability remained largely uneffected. These results show that there was a toxic effect of the pH-medium in itself, but the Al toxic medium showed an additional toxicity.

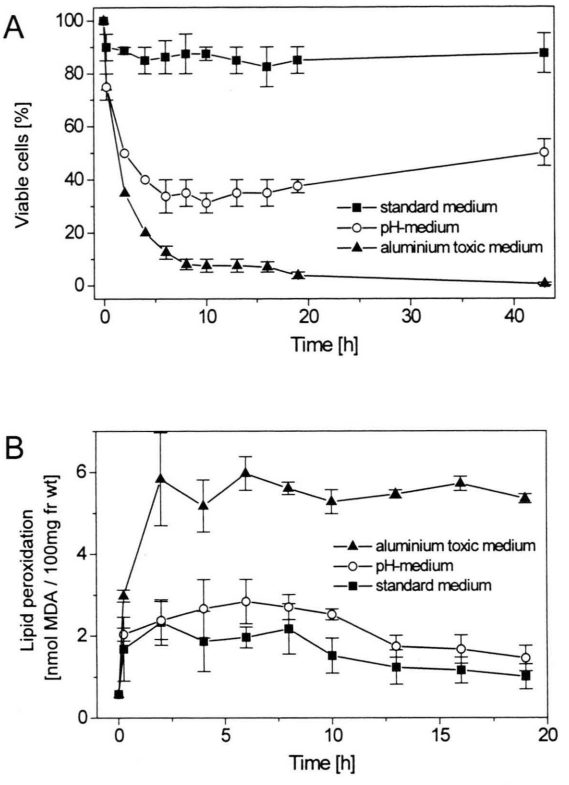

Fig. 1. Cell viability (A) and lipid peroxidation (B) in soybean cells after transfer to standard medium (pH 5.0), pH-medium (pH 4.0) and aluminium toxic medium (pH 4.0, Al^{3+} activity: 15 μM). Cells were washed once in pH-medium and then 1 g fr wt of cells was transferred to 40 ml of the respective media. At the given times lipid peroxidation was measured by the TBA test for MDA and cell viability was determined using FDA and phenosafranine staining. Bars represent S. E. from three (B) or two (A) replicates.

When lipid peroxidation was measured the results were again different for the three media (Fig. 1B). In standard medium a rapid but slight and transient increase in lipid peroxidation to up to 2 nmol MDA/100 mg fr wt was measured by the TBA test. Upon transfer to pH-medium cells showed a similar but slightly stronger transient increase in lipid peroxidation to up to nearly 3 nmol MDA/100 mg fr wt after 6 hours. In Al toxic medium lipid peroxidation of the cells rapidly increased to about 6 nmol MDA/100 mg fr

wt after 4 hours and remained on that level until the final measurement after 19 hours. Essentially as observed for cell viability the Al toxic medium also showed a specific effect on lipid peroxidation. In this case the effect of the pH-medium was less pronounced compared to the standard medium.

Since the incorporation of aluminium sulfate was the only difference between the pH-medium and the aluminium toxic medium the specific effects of the latter on lipid peroxidation must have been caused by either the aluminium or the sulfate component. Therefore, cells were transferred to pH-medium incubated for 24 hours and then 48 μmol sulfate were applied to the medium by adding 1 ml of either potassium sulfate solution (pH 4.0) or aluminium sulfate solution (pH 4.0), respectively. The data given in Table I show that the addition of potassium sulfate did not result in an increase in lipid peroxidation with values of about 3.5 nmol MDA/100 mg fr wt after 6 hours and 2.5 nmol MDA/100 mg fr wt after 24 hours whereas the addition of aluminium sulfate led to an increase up to 6.5 nmol MDA/100 mg fr wt after 24 hours.

The effects of Al were critically dependent on the presence of Fe^{2+} in the culture medium, but Fe^{2+} alone was not capable of increasing lipid peroxidation and reducing cell viability (data not shown).

Taken together these results show that in the presence of Fe^{2+} toxic Al ions led to an inreased lipid peroxidation and a decreased viability of soybean cells in suspension culture. The similar time scales of the two effects suggest a causal relationship between cell death and enhanced lipid peroxidation. However, from these data it was not possible to decide which one was the cause and which one was the consequence.

The effect of lipophilic antioxidants on lipid peroxidation and cell viability

In order to address the question of cause or consequence of Al toxicity for lipid peroxidation we used the lipophilic antioxidants DPPD and BHA that are known to suppress lipid peroxidation (Halliwell and Gutteridge, 1989). When cells were transferred to Al toxic medium supplemented with DPPD (final concentration: 20 μM) dissolved in DMSO cell viability after 16 hours was still about 90% and hardly any lipid peroxidation could be detected by the TBA test for MDA (Fig. 2A). In contrast, after 16 hours in Al toxic medium without DPPD lipid peroxidation had reached 3.5 nmol MDA/100 mg fr wt and cell viability was reduced to 20%. The solvent DMSO alone failed to show a strong effect on both parameters. Another lipophilic antioxidant, BHA, gave similar results at a concentration of 20 μM (Fig 2B). Thus, BHA and DPPD were able to suppress lipid peroxidation in soybean cells in the presence of toxic Al ions which under these conditions also did not effect cell viability.

To further elucidate the effect of the lipophilic antioxidants we tested different concentrations of DPPD. The results presented in Fig. 3 show that the viability of the cells was less effected by Al with rising concentrations of DPPD whereas lipid peroxidation was increasingly suppressed. This effect was already clearly detectable at DPPD concentrations as low as 1–2 μM (Fig. 3A). Thus, the effect of DPPD on lipid peroxidation and viability

Table I. Effect of aluminium sulfate ($Al_2(SO_4)_3$*$18H_2O$) and potassium sulfate (K_2SO_4) on lipid peroxidation in soybean cell suspension cultures.

Duration of treatment	Lipid peroxidation (nmol MDA/100 mg fr wt)	
	After application of Al-sulfate	After application of K-sulfate
0.15h	4.78 ± 0.1	3.41 ± 0.12
6h	6.03 ± 0.53	3.71 ± 0.06
24h	6.5 ± 0.31	2.48 ± 0.1

After transfer of 1 g fr wt of cells to 40 ml of pH-medium and 24h of incubation the culture medium was supplemented with 48 μmol $SO_4$2- by addition of 1 ml of a solution of K_2SO_4 (pH 4.0) or $Al_2(SO_4)_3$*$18H_2O$ (pH 4.0), respectively. Lipid peroxidation was determined by the TBA test for MDA at the given times after addition of $SO_4$2-. The values are means ± S. E. of results from two replicates.

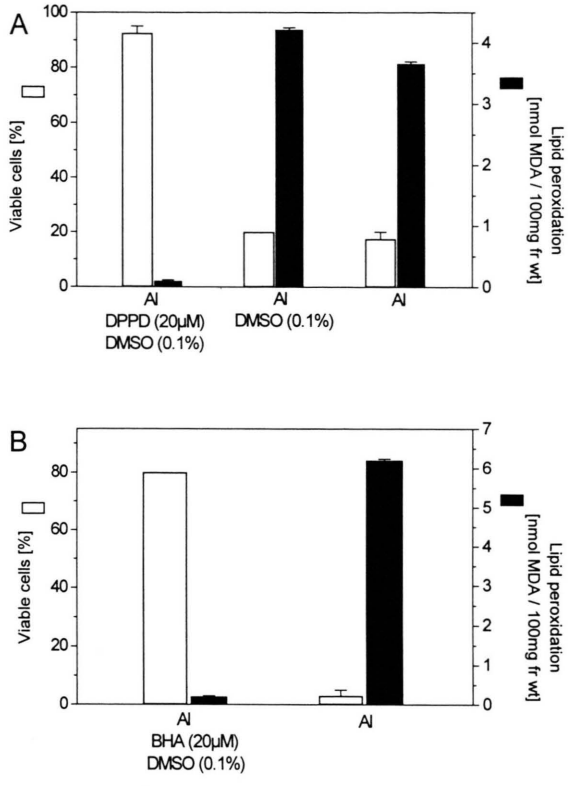

Fig. 2. Effect of the lipophilic antioxidants DPPD (A) and BHA (B) on lipid peroxidation and viability of soybean cells in aluminium toxic medium. Cells were washed once in pH-medium and then 1 g fr wt of cells was transferred to 40 ml of aluminium toxic medium (Al^{3+} activity: 15 µM) with or without DPPD or BHA at a concentration of 20 µM. Since DPPD and BHA were added to the media from DMSO solutions, the effect of DMSO alone was tested at a concentration of 0.1% (v/v). After 16h of incubation lipid peroxidation was measured by the TBA test for MDA and cell viability was determined using FDA and phenosafranine staining. Bars represent S. E. from two replicates.

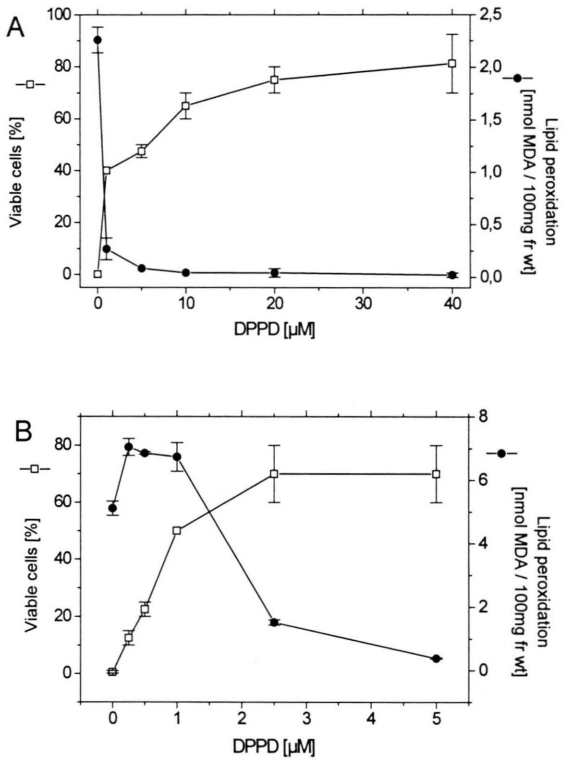

Fig. 3. Dose dependency of the effect of the lipophilic antioxidant DPPD on lipid peroxidation (–●–) and viability (–□–) of cells in aluminium toxic medium. Cells were washed once in pH-medium and then 1 g fr wt of cells was transferred to 40 ml of aluminium toxic medium (Al^{3+} activity: 15 µM) with different concentrations of DPPD: (A) 0–40 µM, (B) 0–5 µM. After 16h of incubation lipid peroxidation was measured by the TBA test for MDA and cell viability was determined using FDA and phenosafranine staining. Bars represent S. E. from two replicates.

of soybean cells in Al toxic medium was dose-dependent.

The results from our experiments with lipophilic antioxidants suggest that cell death in Al toxic medium was a consequence rather than a cause of enhanced lipid peroxidation.

Discussion

Aluminium phytotoxicity is expressed at the cellular level but information on the effects of Al on cultivated plant cells is still scarce (Taylor, 1995). This may be due to the complex chemistry of Al in solution that necessitates important modifications of the culture medium in order to present toxic activities of Al^{3+} (Conner and Meredith, 1985). In plant roots mature cells are not very sensitive to Al but the actively growing cells at the root tip are (Ryan *et al.*, 1993). Similarly, cultivated cells in the stationary phase are less sensitive than cells from exponential or logarithmic growth phases (Yamamoto *et al.*, 1995). Vitorello and Haug (1996) could show that short-term Al uptake by cultured tobacco cells was critically dependent on cell growth. In order to study toxic effects of Al on soybean suspension cells we therefore chose actively growing cells for our experiments.

A number of recent findings point at the plasma membrane as a primary target of aluminium toxicity (Barceló *et al.*, 1996). In soybean rot tips and cultivated cells Al treatment induced callose deposition (Horst *et al.*, 1992; Stass and Horst, 1995). Al-induced callose formation is a well-known marker for Al injury in plant roots and can be used for screening for Al sensitivity (Wissemeier *et al.*, 1992). Activation of callose synthase seems to require elevated cytosolic Ca^{2+}-concentrations and membrane damage (Kauss, 1987). Thus, it is reasonable to assume that Al impairs plasma membrane integrity in soybean cells. Al^{3+} can bind to the plasma membrane and thereby induce alterations of its properties, e.g. membrane fluidity (Deleers *et al.*, 1986; Akeson *et al.*, 1989). The stimulating effect of Al on Fe^{2+}-induced lipid peroxidation is well documented (Gutteridge *et al.*, 1985). When Horst and co-workers assessed lipid peroxidation in Al-treated cultivated soybean cells and soybean roots they could measure an enhanced lipid peroxidation only in the roots but not in the cells that were cultured in a simple $CaCl_2$ solution supplemented with sucrose and $AlCl_3$ (Horst *et al.*, 1992; Stass and Horst, 1995). Based on these results they concluded that the enhanced lipid peroxidation they detected in soybean roots was a consequence of, rather then a reason for, the postulated modification of membrane properties (Stass and Horst, 1995). On the other hand the group of Yamamoto and Matsumoto, who measured enhanced lipid peroxidation caused by a combination of Fe^{2+} and Al^{3+} in tobacco cell cultures, concluded that this oxidative damage to the plasma membrane led to a loss of cell viability (Ono *et al.*, 1995; Yamamoto *et al.*, 1997). They also found that the sensitivity of cultured tobacco cells to Al depended on the solution used for Al treatment. Al-induced lipid peroxidation and cell death only occured in Al toxic nutrient medium, but not in $CaCl_2$ solutions (Yamamoto *et al.*, 1996). It may be hypothesized that the Ca^{2+} concentration in the $CaCl_2$ solution was high enough to prevent effective binding of Al^{3+} to the plasma membrane surface and consequently protected the membrane from lipid peroxidation. The ameliorative effect of elevated Ca^{2+} concentrations on Al toxicity has well been characterized (Rengel, 1992).

The results of our experiments with soybean cell suspensions in Al toxic nutrient medium clearly demonstrate that Al is toxic to soybean cells and that it stimulates Fe^{2+}-induced lipid peroxidation (Fig. 1). The measured values for lipid peroxidation of about 2–6 nmol MDA/100 mg fr wt correspond very well with those found for soybean roots (2 nmol MDA/100 mg fr wt; Horst *et al.*, 1992) and tobacco cells (1.5–6 nmol MDA/100 mg fr wt; Yamamoto *et al.*, 1996; 1997). The use of pH-medium allowed to separate Al specific effects from those caused by low pH, low contents of phosphate and calcium and the absence of EDTA. We can, therefore, say that these modifications impaired cell viability to a certain degree and caused a slight and transient increase in lipid peroxidation (Fig. 1). Nonetheless, there was a distinct specific effect of Al on both parameters.

The enhancement of lipid peroxidation we measured in Al-treated soybean cells could simply be a consequence of the observed cell death, but it could as well be the cause of it. The results of our experiments with lipophilic antioxidants strongly suggest that the latter assumption is correct. DPPD and BHA can effectively donate a hydrogen atom to a peroxy or alkoxy radical, thus blocking the propagation of lipid peroxidation. Thereby they become radicals themselves but these radicals are not sufficiently reactive to support the chain reaction (Halliwell and Gutteridge, 1989). DPPD and BHA were capable of suppressing the Al-induced enhancement of lipid peroxidation in soybean cell cultures. Moreover, in the presence of DPPD and BHA cell death in Al toxic medium was effectively prevented (Fig. 2). The effect of DPPD was dose-dependent and became detectable at concentrations as low as 1–2 μM (Fig. 3). Yamamoto *et al.* (1996; 1997) obtained similar results from experiments with Al-treated tobacco cells. They could detect distinct protective effects of DPPD at a concentration of 0.1 μM. One could argue that DPPD and BHA possess Al binding capacities and lower the activity of Al^{3+}, thus reducing Al toxicity. However, the very low effective doses of DPPD and the chemical structure of both antioxidants do not favour this assumption (Halliwell and Gutterdige, 1989; Yamamoto *et al.*, 1997). We conclude that the suppression of Al-enhanced lipid peroxidation protected the soybean cells from cell death in the Al toxic medium. This means that enhanced lipid peroxidation is responsible for Al-induced death of soybean cells. Espe-

cially under prolonged exposure to Al other toxic effects may also contribute to Al-induced cell death, but in our short-term experiments lipid peroxidation seems to be the major factor.

Unlike Fe^{2+} and reactive oxygen species Al^{3+} can not directly initiate peroxidation of lipids (Halliwell and Gutteridge, 1989). Furthermore, in our experiments Al ions in the culture medium were not capable of inducing the formation of reactive oxygen species that could have initiated lipid peroxidation (data not shown). According to Oteiza (1994) we therefore suppose that rearrangments in the lipid bilayer induced by Al^{3+}-binding to the hydrophilic phospholipid head groups rendered the membrane lipids more susceptible to Fe^{2+}-induced lipid peroxidation (Oteiza, 1994).

If we assume that lipid peroxidation is a decisive factor for Al toxicity in soybean cells the question remains how lipid peroxidation would cause cell death. There are at least three possible explanations: (i) It was recently shown that MDA and other lipid peroxidation products can be toxic to plant cells when applied exogenously (Adams *et al.*, 1999). Thus, lipid peroxidation products themselves could cause intracellular damage and thereby mediate Al toxicity. (ii) Al-induced enhanced lipid peroxidation could lead to a massive loss of membrane integrity that would directly lead to cell death (Ikegawa *et al.*, 1998). (iii) Lipid peroxidation could facilitate the entry of Al into the cytoplasma where several Al-sensitive targets are located (Kochian, 1995). Further examinations are required to elucidate the possible involvement of any of these mechanisms in Al-induced cell death. Recent results that reveal similarities between Al cytotoxicity and apoptosis (Yamaguchi *et al.*, 1999) offer an interesting new direction for future research.

Acknowledgements

This study was supported by the German Federal Ministry of Education and Research (BMBF, Bonn & Berlin) and Fonds der Chemischen Industrie, Frankfurt.

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