Aluminum Toxicity in Soybean Cell Cultures

Ingo Rath

Institute of Plant Biochemistry and Biotechnology, University of Münster, Germany

ABSTRACT

Aluminum phytotoxicity is a major factor of the infertility of acid soils. The main toxic form of aluminum is the Al^{3+} ion. Its occurrence strongly depends on the pH of the solution and the activity of other ions that can interact with Al^{3+}. Simulating the mineral environment of aluminum-toxic soils in plant cell culture requires careful modifications to the standard culture medium. In principle, using such an aluminum-toxic medium it is possible to select for cell lines that show an increased resistance to aluminum. Selection for aluminum resistance can be achieved by different strategies. In direct strategies cells are selected for their ability to actively grow in the presence of toxic concentrations of ionic aluminum. Rescue strategies involve a recovery of cells in normal medium that have survived a previous aluminum treatment. Different direct and rescue methods and required medium modifications for soybean cell cultures are presented and discussed with regard to arising problems and possible solutions. Aluminum ions cause a multitude of harmful changes to various cellular process. Despite intensive research a primary target of aluminum phytotoxicity has not been identified so far. Al^{3+} can enhance the iron-mediated peroxidation of membrane lipids. In soybean cell cultures this stimulation of lipid peroxidation coincides with aluminum-induced cell death. Experiments with lipophilic antioxidants showed that soybean cells survived in the presence of cytotoxic levels of aluminum in the culture medium when lipid peroxidation was suppressed. This leads to the conclusion that aluminum toxicity in soybean cell cultures is at least partly mediated by an enhancement of lipid peroxidation. The underlying mechanism of Al^{3+} action could involve the increased formation of reactive oxygen species. In soybean cell cultures, however, aluminum treatment did not lead to a measurable formation of reactive oxygen species. Therefore, it seems more likely that direct interactions between Al^{3+} and membrane lipids lead to changes in membrane structure that facilitate lipid peroxidation processes. The importance of different toxic effects of aluminum on soybean cells are discussed.

Key words: Aluminum toxicity, *Glycine max*, cell culture

SELECTION OF ALUMINUM-RESISTANT SOYBEAN CELL LINES: PROBLEMS AND SOLUTIONS

Acid soils are found in many regions all around the world (Figure 1) (von Uexküll and Mutert, 1995). Probably the major hindrance to agricultural use of these soils is the phytotoxicity of aluminum under acidic conditions. There are two different strategies to overcome this problem. Liming and the use of fertilizers can ameliorate the soil quality and the use of aluminum-resistant crop plants can lead to increased yields. In addition to conventional breeding programs *in vitro*-selection of aluminum-resistant cell lines and subsequent regeneration can be used to gain improved crop plants (Parrot and Bouton, 1990; Van Sint Jan *et al.*, 1997). If this basic approach is chosen, the complex chemistry of aluminum in solution necessitates a careful control of culture medium

parameters to make sure that aluminum toxicity can be generated in cell culture (Conner and Meredith, 1985a; Wersuhn *et al.*, 1994). The main problem in doing so is to ensure that aluminum ions in the selective culture medium are not precipitated and that they are present in a toxic form (Taylor, 1995). There are four modifications to the composition of commonly used cell culture media that have to be made to achieve this.

1. The pH-value of the culture medium has to be adjusted and maintained below 5. Only under these acidic conditions aluminum will be present predominantly as the toxic Al^{3+} ion (Figure 2) (Macdonald and Martin, 1988). Because of its balanced ratio of NO_3- and NH_4^+ the MS medium (Murashige and Skoog, 1962) is regarded as the most appropriate basal medium for the following modifications (Conner and Meredith, 1985a; Marziah, 1991).
2. The iron chelator EDTA has to be omitted. EDTA can also chelate Al^{3+} and thereby detoxify it (Figure 3), because the resulting Al-EDTA complex is not toxic to plant cells (Conner and Meredith, 1985a).
3. The phosphate content of the culture medium has to be reduced. Phosphate is able to precipitate Al^{3+} by forming barely soluble Al-phosphate (Figure 3). This drastically reduces the activity of the toxic Al^{3+} ion (Conner and Meredith, 1985a).

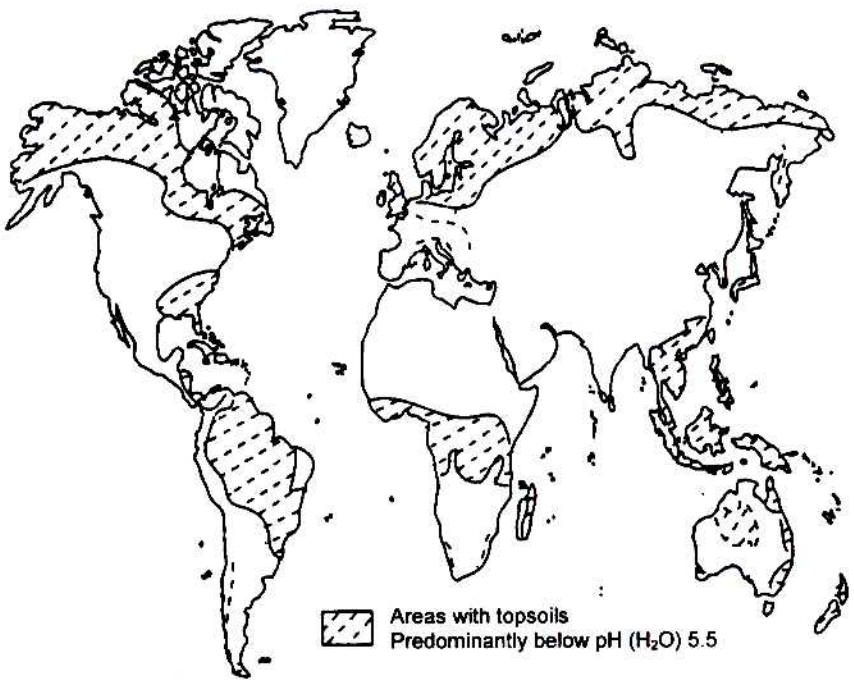


Figure 1. World wide distribution of areas with topsoils predominantly below pH 5.5
source: von Uexküll and Mutert, 1995

4. The calcium content of the culture medium may have to be lowered. Ca^{2+} is a known antagonist to Al^{3+} and can alleviate many aluminum toxicity symptoms (Figure 3). High calcium concentrations may therefore counteract toxic action of aluminum upon the cultured cells (Rengel, 1992).

Such a modified culture medium (pH 4.0, 0.1 mM Ca, 0.01 mM P, no EDTA) containing up to 0.8 mM Al (provided as $Al_2(SO_4)_3 18H_2O$) is currently being used in our lab for selection of aluminum-resistant soybean cell lines.

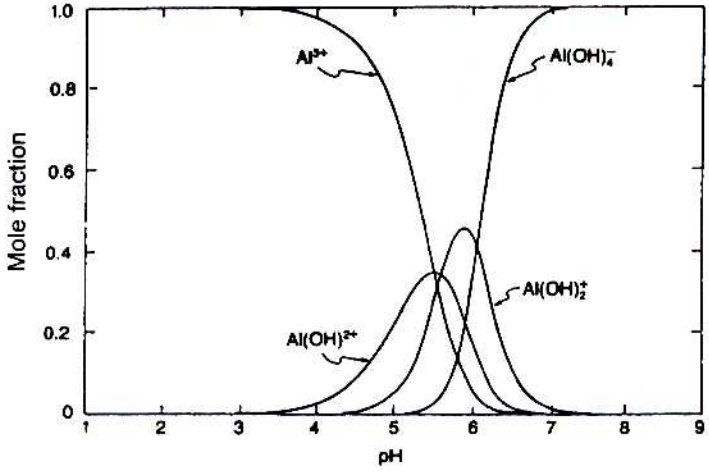
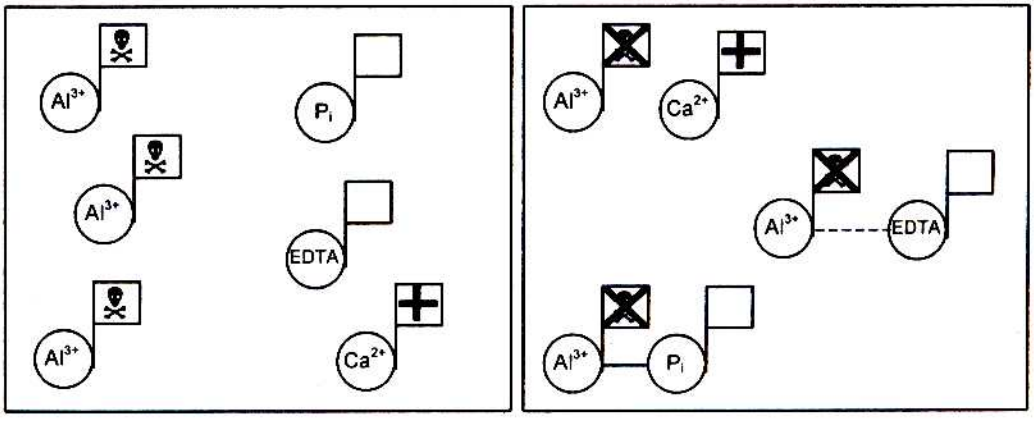


Figure 2. Distribution of soluble, mononuclear aluminum ion species in water as a function of pH (after Macdonald and Martin, 1988)

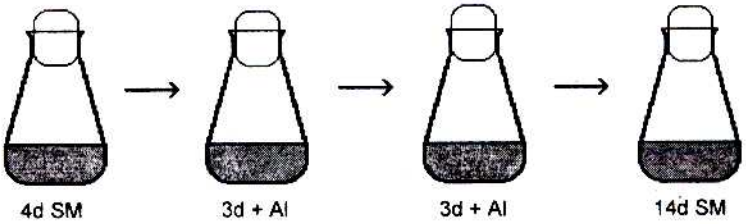


Notes: Calcium (Ca^{2+}) is an antagonist to aluminum and can counteract its toxic effects, EDTA detoxifies aluminum by chelation, phosphate (P_i) precipitates aluminum and thereby removes it from solution

Figure 3. Possible interactions between toxic aluminum (Al^{3+}) and other culture medium components that reduce aluminum toxicity

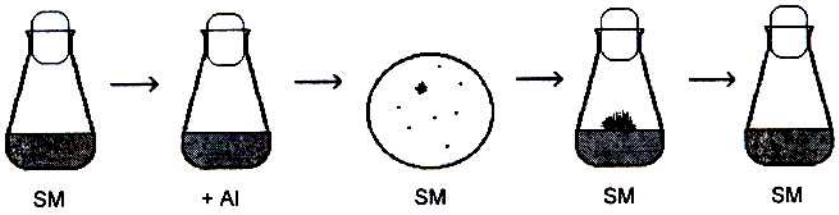
The selection procedure can comprise different subculture strategies that will be discussed below. Since in cell culture as well as in roots only actively growing cells show sensitivity to aluminum it is recommended to use only cells from the growth phase of the batch culture and not from lag phase or stationary phase (Yamamoto *et al.*, 1994; 1995).

1. Permanent culture in liquid selective (Al-containing) medium. This is not appropriate because the medium modifications described above do not support a prolonged cultivation.
2. Alternating subculture in liquid selective and non-selective medium. This allows the recovery and propagation of cells that survived a selection step before starting the next step. The stability of the culture medium parameters may be increased by transfer of the cells to fresh selective medium every two or three days during the selective step (Figure 4). Problems can arise from the low density of surviving cells and because it is not possible to separate different clones.
3. Plating cells on solidified non-selective medium after selection step in liquid selective medium (Conner and Meredith, 1985b). This method allows separation and growth of different surviving clones even at a low density and the use of conditioned medium to stimulate cell growth. The obtained calli can be used to get suspension cultures and continue the selection program (Figure 5).



Notes: Time of subculture duration is given in days (d), SM = standard medium, + Al = selective medium containing aluminum

Figure 4. Illustration of divided selection step using liquid selective medium



Notes: SM = standard medium, + Al = selective medium containing aluminum

Figure 5. Illustration of selection step involving recovery of plated cells

4. Plating cells directly on solidified selective medium (Arihara *et al.*, 1991). This is a more stringent version of method C. Control of medium parameters by frequent subculture is complicated. For effective growth, surviving clones may have to be transferred to non-selective solidified medium before suspension cultures can be obtained from them.
5. Use of callus cultures instead of suspension cultures with method D (Van Sint Jan *et al.*, 1997). This may allow a more effective action of those mechanisms of aluminum resistance that depend on the formation of a micro-environment (e.g. excretion of organic acids, pH-shifts in the apoplast). However, there is a risk of selection of a chimeric cell line consisting of several clones that differ in aluminum resistance (Collin and Dix, 1990).

Methods A and B were not successful with soybean cell cultures from the cultivars Doko RC and Wilis. With method C a number of clones have been obtained from both cultivars but none of them showed a stable and reproducible aluminum resistance (Rath, 1999). Our current work is focussed on methods C and D. Using the latter some clones could be selected and their level of aluminum resistance is under investigation.

In summary, the selection of aluminum-resistant plant cell lines using cell cultures is a difficult task that requires careful control of different medium and subculture parameters. Some reports on the successful selection of Al-resistant cell lines are questionable, because of a lack of control of these parameters (Ojima and Ohira, 1983; Smith *et al.*, 1983; Wersuhn *et al.*, 1988). Only a very limited number of *in vitro*-selection experiments for Al-resistance have without a doubt led to the desired clones with a stable resistance (Conner and Meredith, 1985a; 1985b; 1985c; Ojima *et al.*, 1989; Arihara *et al.*, 1991; Van Sint Jan, 1997).

MECHANISMS OF ALUMINUM TOXICITY IN SOYBEAN CELL CULTURES

Aluminum toxicity is expressed in cell cultures as well as in root tips of plants (Taylor, 1995). The underlying mechanisms are still poorly understood. Many possible toxic effects of Al^{3+} are known but the primary target of Al-toxicity remains elusive. It is known that aluminum can bind to the plasma membrane and thereby alter its physical properties (Akeson *et al.*, 1989; Caldwell, 1989). Some recent reports indicate that there may be a correlation between aluminum phytotoxicity and non-enzymatic Fe^{2+}-induced membrane lipid peroxidation (Figure 6) (Ono *et al.*, 1995; Yamamoto *et al.*, 1996; 1997). In soybean cell cultures lipid peroxidation is rapidly enhanced by aluminum ions in the culture medium. Simultaneously, the viability of the cells declines (Figure 7). These results clearly show that the two phenomena are linked, but no conclusion can be drawn about which one is the cause and which is the effect.

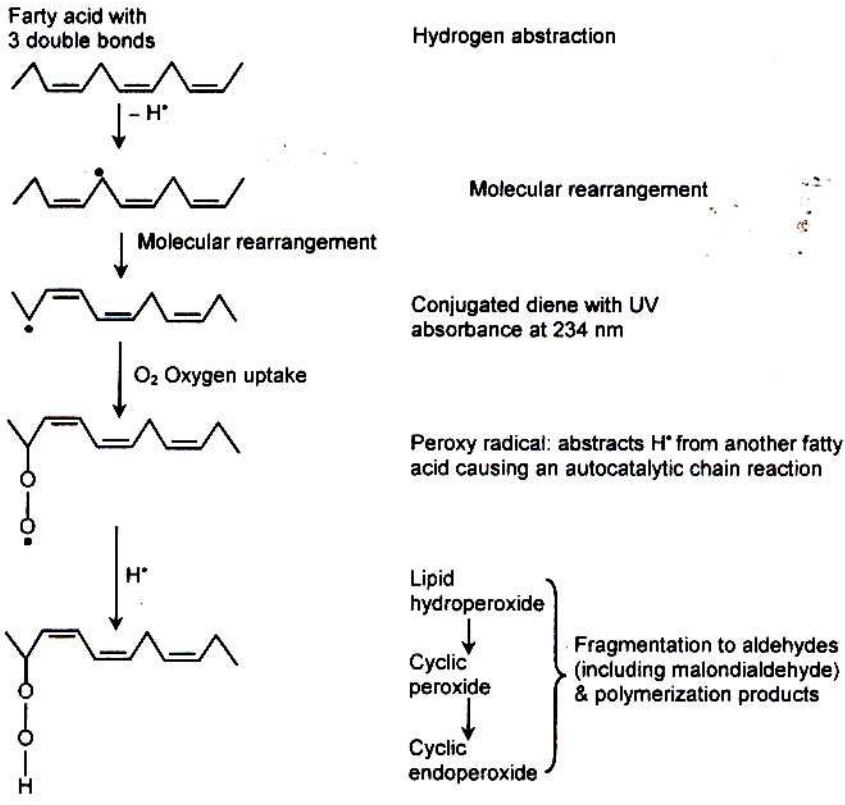
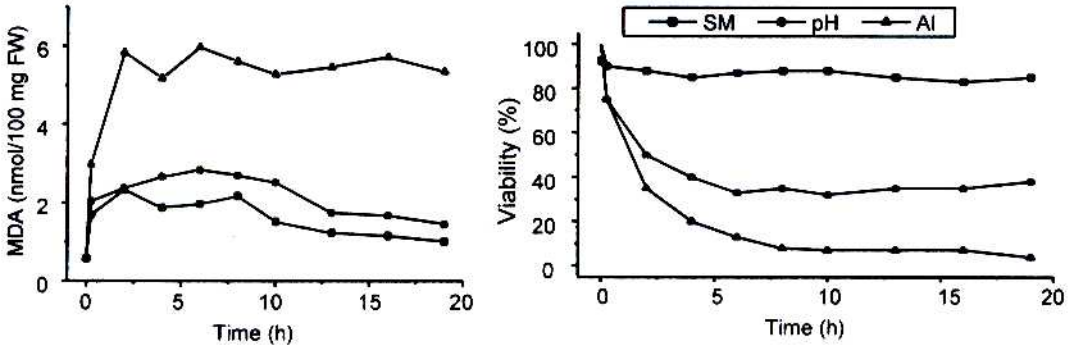


Figure 6. Idealized representation of the initiation and propagation reactions of lipid peroxidation (after Halliwell and Gutteridge, 1989)

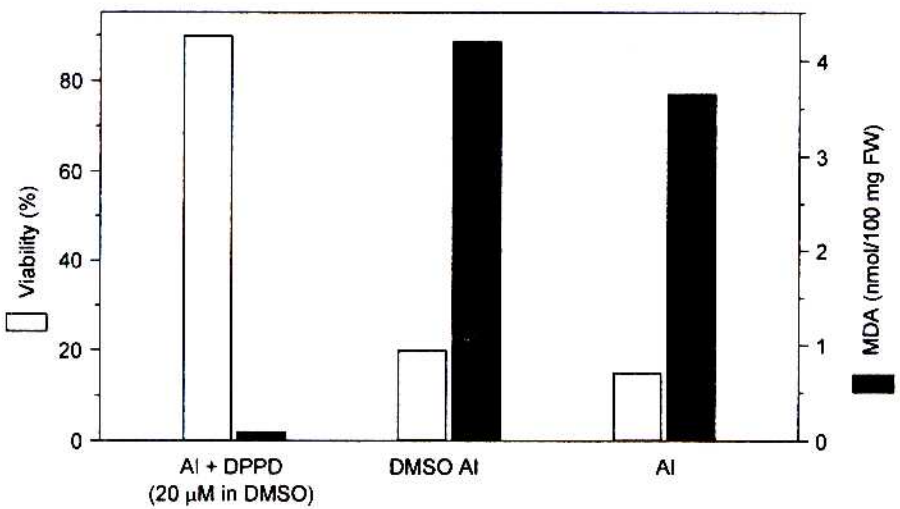


Notes: Viability was determined by dye stainings. Lipid peroxidation was measured using the TBA test for malondialdehyde (MDA)

Figure 7. Time course of lipid peroxidation (top) and viability (bottom) of soybean cells after transfer to standard medium (SM), acid medium (pH), and aluminum-toxic medium containing 0.8 mM Al (Al)

To further elucidate the relation between Al-toxicity and Al-induced lipid peroxidation experiments with lipophilic antioxidants that can suppress lipid peroxidation were conducted. When *N,N'*-diphenyl-*p*-phenylene diamine (DPPD) was added to aluminum-toxic culture medium cells that were transfered into this medium did not exhibit an increased lipid peroxidation and remained viable (Figure 8). The same effect could be achieved by the addition of other lipophilic antioxidants. The suppression of lipid peroxidation largely abolished aluminum toxicity in soybean cell cultures. This proves that aluminum toxicity is at least partly mediated by an enhancement of lipid peroxidation leading to cell death.

There is still some confusion about the way that aluminum ions can stimulate lipid peroxidation. It was proposed that they might lead to the generation of reactive oxygen species (ROS) that would increase lipid peroxidation (Cakmak and Horst, 1991). In soybean cell cultures no Al-specific generation of ROS could be detected. Thus, it seems more likely, that aluminum ions induce the formation of tight clusters of membrane lipids by binding to their polar head groups. Within these clusters the initiation and propagation of lipid peroxidation is facilitated (Oteiza, 1994). Further work is needed to elucidate the precise relationship between toxic aluminum ions, lipid peroxidation, and cell death in soybean cells.



Notes: 16 h after transfer to aluminum-toxic medium containing 0.8 mM Al and DMSO or DPPD (in DMSO) cells viability was determined by dye stainings and lipid peroxidation was measured using the TBA-test for malondialdehyde (MDA)

Figure 8. Effect of the lipophilic antioxidant *N,N'*-diphenyl-*p*-phenylene diamine (DPPD, 20 µM) and its solvent dimethylsulphoxide (DMSO, 0.1%) on lipid peroxidation and viability of soybean cells in the presence of toxic aluminum

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