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Rapid and simple method for Al-toxicity analysis in emerging barley roots during germination

L. TAMÁS*, S. BUDÍKOVÁ, M. ŠIMONOVIČOVÁ, J. HUTTOVÁ, B. ŠIROKÁ and I. MISTRÍK

Institute of Botany, Slovak Academy of Sciences, Dúbravská cesta 14, SK-84523 Bratislava, Slovak Republic

Abstract

The results demonstrate the benefits of using filter-paper-based system for cultivation the germinating barley seeds for Al toxicity or Al tolerance analyses. Due to the high affinity of filter paper to Al monomeric forms, milimolar Al concentrations were required to cause similar Al toxicity symptoms of roots as micromolar Al concentrations in hydroponics: 1 mM Al had no effects on the emerging barley roots, 2 mM Al was moderately toxic but roots showed good recovery, 4 mM Al was highly toxic and 8 mM Al even lethal. Screening of eight barley cultivars revealed different rank of their tolerance to Al. The root growth inhibition positively correlated with the Al concentration in root tips.

Additional key words: aluminium, cell viability, cultivation method, hematoxylin.

Introduction

Several cultivation methods have been used to analyse Al toxicity. Soil culture is on the one hand the most natural environment for plants; on the other hand, several disadvantages could emerge while using it for Al toxicity analyses. One of them is the inability to ensure the same quantitative and qualitative composition of soil samples in repeated experiments. Moreover, problems with observing the intact roots and root tips in soil culture are intractable. To avoid these obstacles, soil cultures were substituted by sand cultures in some experiments (Villagarcia *et al.* 2001).

Hydroponics is very frequently used method in Al toxicity experiments; using simple $CaCl_2$ solution, it was applied in screening for Al tolerance of many plants including barley (Ma *et al*. 1997). Disadvantage of using this cultivation technique is that the transfer of young germinated barley seedlings to hydroponics can cause significant stress and requires a long period for the onset of several mechanisms to adapt to hydroponics, *e.g*., metabolic changes and sometimes aerenchyma formation. Therefore, analysis of Al toxicity during germination and in young seedlings is impossible in hydroponics, especially in plants sensitive to hypoxia. In addition, the water film adhering to the root, which plays a crucial role in pH regulation of root surface, is disturbed in hydroponics (Felle 1998). All these factors might lead to intensifying of the sensitivity of root tissues to Al in comparison with plants cultivated in soil.

To avoid the disadvantages of above-mentioned methods we conducted a set of experiments focused on the toxicity of Al in young barley seedlings using the filter paper technique for germination and early growth of roots. Germination and cultivation of seedlings has been performed between two layers of filter paper (*Whatman No 1*) moistened with solutions of appropriate Al concentration.

Materials and methods

Plants and growth conditions: Caryopses of barley (*Hordeum vulgare* L.) cv. Alfor, or in screening experiments cvs. Express, Jubilant, Madonna, Progress, Kompakt, Ludan and Bavaria were surface sterilised with 12 % H_2O_2 for 10 min and then rinsed three times for 10 min with distilled water. After 4 h of incubation in 8.2 mM $CaCl_2$ solution, pH 4.0 (control), or in 7.2, 6.2, 4.2 or 0.2 mM $CaCl_2$ containing 1, 2, 4, and 8 mM $AlCl_3$

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* Corresponding author; fax: (+421) 2 54771948; e-mail: ladislav.tamas@savba.sk

solution, pH 4.0 (Al-treated) at 25 °C in darkness the caryopses were germinated between two layers of filter paper (*Whatman No 1*) fully moistened with the same solutions and at the same conditions. After 20 h from the onset of experiments the germinating caryopses were transferred on the freshly moistened filter papers with appropriate solutions. In recovery experiments seedlings after 48 h of Al treatment were transferred to fresh filter paper moistened with 8.2 mM $CaCl_2$ solution, pH 4.0. Root length was measured with a ruler, and seedlings were used immediately for analysis. Each experiment was repeated at least five times with 60 seedlings.

Aluminium accumulation: Hematoxylin staining was used for determination of Al uptake (Ownby 1993). Freshly harvested roots after washing in distilled water for 15 min were stained with 0.2 % hematoxylin (m/v) and 0.02 % KIO_3 (m/v) solution for 15 min at room temperature. After rinsing with distilled water for 15 min, root tips were cut for light microscopy observation or 10 root tips (5 mm) were excised and soaked in 0.2 cm^3 of 1 M HCl for 1 h. The absorbance of released stain was measured at 490 nm by spectrophotometer (*Beckman DU-8B*, Fullerton, USA).

Determination of cell viability: The loss of cell viability was evaluated using Evans blue staining method (Baker and Mock 1994). Freshly harvested roots were stained with 0.25 % (m/v) aqueous solution of Evans blue for 15 min. After rinsing with distilled water for 30 min, root tips were cut for light microscopy observation or 10 root tips (5 mm) were excised and soaked with 0.2 cm^3 of N,N-dimethylformamide for 1 h at room temperature. The absorbance of released Evans blue was measured spectrophotometrically at 600 nm.

Aluminium determination in solution: The amount of Al bound to the filter paper was determined by measuring the concentration of Al remaining in the solution by aluminon method (Kerven *et al.* 1989). Two filter papers fully moistened in solution containing appropriate Al concentration (both Al-treated and control) were placed into Petri-dish and after 24 h were centrifuged to obtain solution containing soluble Al. 0.2 cm^3 of this sample was added to 0.065 cm^3 aluminon solution (2.4 g NaOH was dissolved in 20 cm^3 deionized water and 12 cm^3 acetic acid was added. 17.5 mg of aluminon was dissolved in this buffer solution and than diluted to 50 cm^3). After 15 min of incubation the absorbance was measured at 530 nm. A standard calibration curve was prepared for the 0 - 250 μM Al concentration range.

Determination of pH: To test, how the caryopses were able to alkalinize the solution applied to the filter paper, the pH value was determined with a pH-meter after 4-h incubation of 100 caryopses in 100 cm^3 8.2 mM $CaCl_2$ solution, pH 4.0 (control), or in solution containing 7.2, 6.2, 4.2 or 0.2 mM $CaCl_2$ and 2, 4, 6, and 8 mM $AlCl_3$, pH 4.0 - 3.6 (Al-treated) at 25 °C in darkness; and after 20 and 48 h - germination of 100 caryopses between two filter papers fully moistened with the same solutions and at the same conditions. After collecting the germinated caryopses, filter papers were moistened in 100 cm^3 deionized water for 15 min, in which the pH was measured after removing the filter papers.

Results

Root growth inhibition: Root growth of barley caryopses germinated between two layers of moistened filter paper was not affected by 1 mM Al during 96 h of cultivation (Fig. 1), while it was significantly inhibited between 48 and 72 h of 2 mM Al treatment. Al at 4 and 8 mM concentration inhibited root growth already between 24 and 48 h from the start of germination. Strong inhibition of root growth was observed 48 h after the onset of seed imbibition at 8 mM Al. In recovery

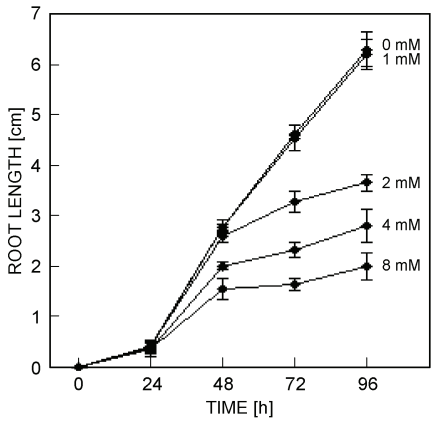

Fig. 1. Root growth inhibition of barley plants grown at 0, 1, 2, 4, and 8 mM $AlCl_3$ for 24, 48, 72 and 96 h. Means ± SD (n = 5).

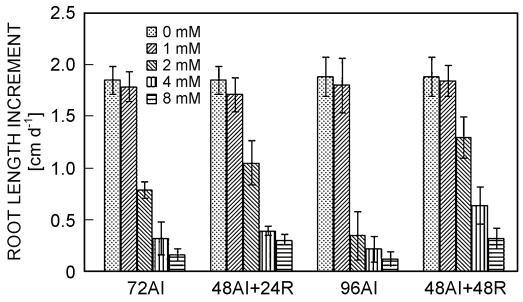

Fig. 2. Root length increment of barley plants grown at 0, 1, 2, 4, and 8 mM $AlCl_3$ and during recovery. 72 h Al-treatment; 48Al+24R = 48 h Al treatment followed by 24 h recovery; 96 h of Al treatment; 48Al+48R = 48 h of Al treatment followed by 48 h recovery. Values represent increment of the last 24 h of root growth. Means ± SD (n = 5).

experiment after 48 h of Al treatment at 8 mM Al concentration, root growth was not restored (Fig. 2), and even at 4 mM Al root growth was restored only slightly and only on the second day of cultivation without Al. Significant root growth during recovery was detected only after applying 2 mM Al, it nearly achieved root growth increment of control plants.

Aluminium uptake: No marked hematoxylin stain was detected by measuring the absorbance in controls and 1 mM Al treated root tips (Fig. 3). Higher Al concentrations (2, 4 and 8 mM) resulted in visible hematoxylin staining already 48 h after the onset of germination, which steadily increased after 72 and 96 h. Light microscopy seemed to be more sensitive to hematoxylin staining; the observations showed faint staining of rhizodermal cells in meristematic region already at 1 mM Al concentration. It intensified after longer treatment (Fig. 4*B*,*B*'). With the increasing concentration to 2 mM the roots accumulated aluminium more readily (Fig. 4*C*,*C*'). Hematoxylin staining was observed in rhizodermal and outer cortical cell walls in the case of 48 h treatment by 4 mM Al (Fig. 4*D*) but with prolonged 72 h treatment and/or 8 mM Al, it penetrated as deep as the endodermis and heavily stained the lumen of peripheral cells (Fig. 4*E*,*D*',*E*'). After 96 h of Al treatment the light microscopy observations showed very similar effects as after 72 h (data not shown).

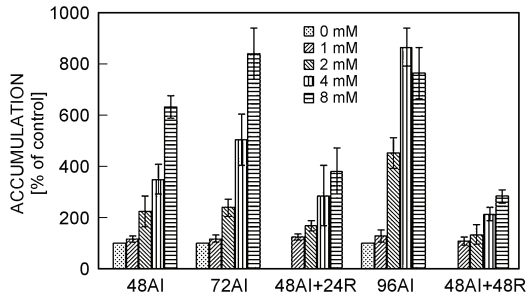

Fig. 3. Accumulation of Al in cells of barley root tips (0.5 cm) stained by hematoxylin grown at 0, 1, 2, 4, and 8 mM $AlCl_3$ for 48, 72 and 96 h, and after recovery (R); 48Al+24R = 48 h Al treatment followed by 24 h recovery; 48Al+48R = 48 h Al treatment followed by 48 h recovery. Control = 100 %. Means ± SD (n = 5).

After 48 h of cultivation in 2, 4 or 8 mM Al the roots were subjected to 24 h recovery treatment. At that time the hematoxylin localisation of Al in roots growing at 2 mM Al was restricted to scattered rhizodermal cells (Fig. 5*K*). In the case of 4 mM Al, the hematoxylin staining vanished from the cortical cells totally (Fig. 5*L*) or it was present in their cell walls only after 8 mM Al and recovery (Fig. 5*M*). Quantitative analysis of the amount of hematoxylin stain in the root tips after recovery period is showed in Fig. 3.

Cell viability: The root tip cell viability at 1 mM Al concentration remained high throughout 48, 72 and 96 h treatments, and only negligible Evans blue uptake was detected (Fig. 6). The accumulation of Evans blue at this Al concentration appeared in the outer cell of rhizodermis only (Fig. 4*G*,*G*'). With the concentration increasing to 2 mM the Evans blue was entrapped in all rhizodermal cells (Fig. 5*H*,*H*') and the amount of Evans blue in root tip cells was about 160, 200 and 240 % of control plants after 48, 72 and 96 h, respectively (Fig. 6). Higher (4 and 8 mM) Al concentrations caused severe stress to root tip inducing the formation of surface lesions reaching the root hair zone and the dead surface cells had sloughed off (4*I*,*I*',*J*,*J*'). These injuries are reflected in rapid rise of Evans blue uptake already 48 h after the onset of seed imbibition (Fig. 6). Despite the fact that both highest concentrations substantially decreased viability of rhizodermal cells, no Evans blue accumulation was found in cortex.

In the roots grown in 2 mM Al for 48 h no higher amount of Evans blue has been detected after subsequent 24 h as well as 48 h of recovery in root tips compared to control plants (Fig. 5*K*', Fig 6). Evans blue uptake decreased rapidly during recovery also in root tip cells growing at higher Al (4 and 8 mM) concentrations (Fig. 6); however, they exhibited serious surface injuries and cell viability loss in rhizodermis (Fig. 5*L*',*M*').

Aluminium binding by filter paper: In hydroponics, μM Al concentrations can be toxic for plants. On filter paper, however, 1 mM concentration still had no effects on germination and early root growth. This apparent discrepancy could be explained by binding of Al to the filter paper. Al concentration decreased in applied solution after binding of Al to cellulose component of filter paper (Table 1). It is evident that 1 and 2 mM Al concentrations were reduced to 170 and 220 μM, respectively.

Table 1. The amount of free available monomeric Al after binding to filter paper (*Whatman No 1*) determined by aluminon method.

Al conc. (solution) Al [mM]	Al conc. (filter paper) A_{530}	Al [μM]
0.0	0.152 ± 0.003	0
0.2	0.245 ± 0.005	37
0.4	0.386 ± 0.005	70
0.6	0.505 ± 0.008	105
0.8	0.660 ± 0.010	145
1.0	0.726 ± 0.006	170
2.0	0.917 ± 0.011	220

Regulation of pH: The pH analysis revealed that control caryopses were able to increase the pH of solution from pH 4 to 6 after 4 h of incubation (Table 2). In contrast, at 1 mM Al the original pH value remained stable, no pH increase occurred. On filter paper, not only germinating control caryopses were able to increase pH from 4 to

about 5.5 during 48 h of cultivation, but also seedlings treated by 1 mM Al elevated pH value to about pH 5. At 2 mM Al, the pH value was increased from 3.8 to about 4.6, while at higher Al concentrations only negligible changes were observed.

Screening of barley cultivars for Al tolerance: To test the applicability of filter-paper-based system for cultivation as a screening system for Al tolerance detection, eight barley cultivars were tested at 2 mM Al

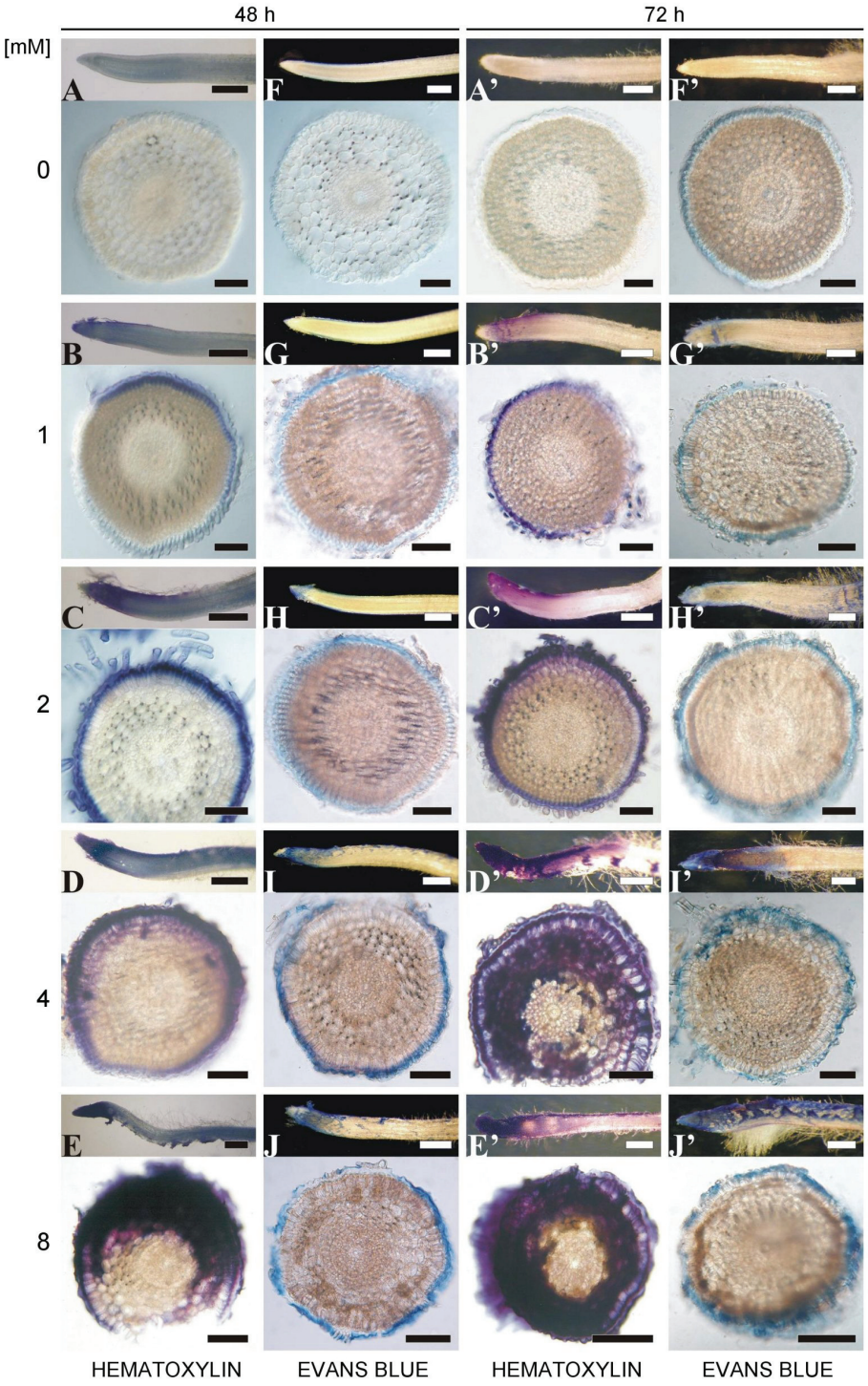

Fig. 4. Localisation of aluminium by hematoxylin, and viability test determined by Evans blue staining in barley roots tips and cells of root transversal section after 48 and 72 h of treatment with different concentration of Al (0, 1, 2, 4, and 8 mM Al). The distance of the sections from the root tip was 1 - 3 mm. *Scale bars* represent 1 mm for root tips and 200 μm for sections.

concentration. The measurement of root growth inhibition revealed different ranking of Al tolerance of analyzed cultivars (Fig. 7*A*). The Al-induced root growth inhibition was positively correlated with the Al uptake of root tips (Fig. 7*B*). On the other hand, no correlation was observed between Al-induced root growth inhibition and Al-induced loss of cell viability (Fig. 7*C*).

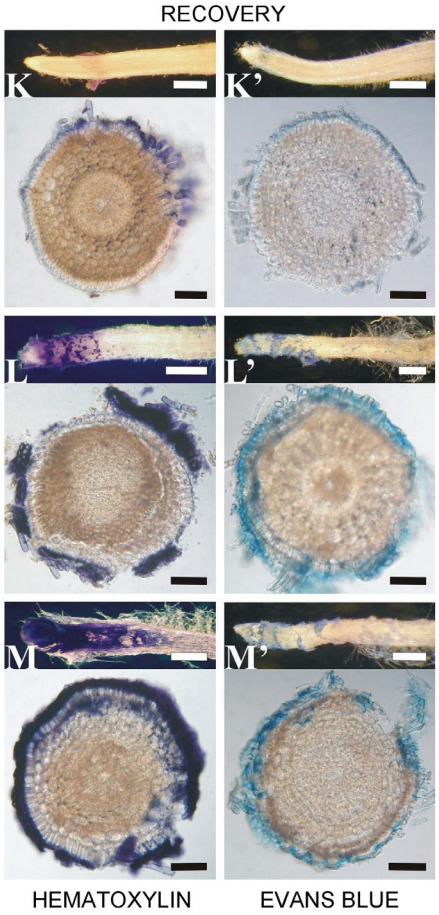

Fig. 5. Localisation of aluminium by hematoxylin, and viability test determined by Evans Blue staining in barley roots tips and cells of root transversal section after 48 h of treatment with different concentration of Al (2, 4, and 8 mM Al) and 24 h recovery experiments. The distance of the sections from the root tip was 1 - 3 mm. *Scale bars* represent 1 mm for root tips and 200 μm for sections.

Table 2. The pH of imbibition solution 0 and 4 h after the start of imbibition and of the solution isolated from filter paper 24 and 48 h after the start of germination.

Al [mM]	Solution 0 h	4 h	Filter paper 24 h	48 h
0	4.00	5.967 ± 0.153	5.333 ± 0.153	5.567 ± 0.153
1	3.90	3.867 ± 0.058	4.967 ± 0.153	5.233 ± 0.058
2	3.80	3.633 ± 0.208	4.567 ± 0.153	4.567 ± 0.208
4	3.65	3.733 ± 0.153	4.100 ± 0.200	4.100 ± 0.100
8	3.60	3.633 ± 0.058	3.900 ± 0.100	4.067 ± 0.058

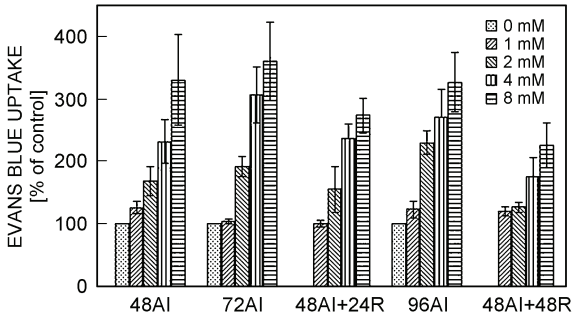

Fig. 6. Accumulation of Evans Blue in cells of barley root tips (0.5 cm) grown at 0, 1, 2, 4, and 8 mM $AlCl_3$ for 48, 72 and 96 h, and after recovery (R); 48Al+24R = 48 h Al treatment followed by 24 h recovery; 48Al+48R = 48 h Al treatment followed by 48 h recovery. Control = 100 %. Means ± SD (n = 5).

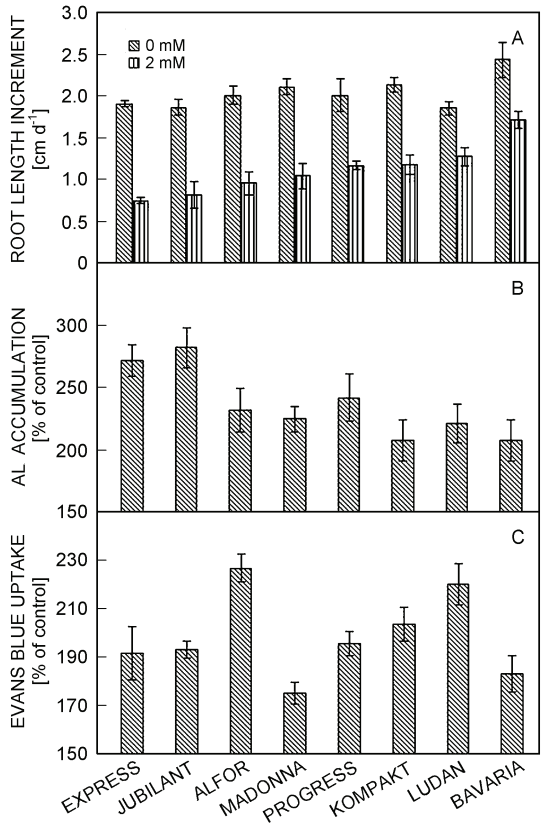

Fig. 7. Screening of barley cultivars for Al tolerance at 2 mM Al concentration. *A* - root length increment of the 24 h growth of barley plants 48 h after germination; *B* - intensity of hematoxylin stain in root tips 72 h after germination; *C* - uptake of Evans blue 72 h after germination.

Discussion

Using filter-paper-based system for cultivation the young barley seedlings seems to be the suitable method for Al toxicity and Al tolerance analyses. Cultivation of seedlings on filter paper was successfully used in investigations of salt-induced changes in rice roots (Lin and Kao 1999) or to study the effects of hormone, drought and cold on barley (Malatrasi *et al.* 2002).

The first reason of using high Al concentrations in our experiments was the binding of Al to the filter paper. It represented 90 % of 2 mM Al concentration and therefore caused the substantial decrease of the free Al^{3+} concentration in the applied solution. For similar reasons, milimolar concentration range of Al was applied in the nutrition medium, where the partial chelating of soluble Al by components of the medium occurred (Pan *et al.* 2001).

Significant injuries of root tip cells were observed in barley cultivated in hydroponics and exposed to 50 μM Al (Budíková and Mistrík 1999). According to this finding and to our results, it seems to be possible that in plants cultivated on filter paper some Al-detoxifying mechanisms are activated, which could be expressed to larger extent compared to hydroponics. One of the suggested mechanisms decreasing Al toxicity is the root-induced alkalization of external media. Increase in the pH value to above pH 4.5 causes rapid conversion of Al from its toxic soluble Al^{3+} form to insoluble non-toxic form. In the soil the fine film of liquid called unstirred layer covers the root surface, in which the pH, ion and organic acid concentration are crucial for transport processes and root growth (Kochian and Lucas 1983, Felle 1998). In natural conditions only torrential rain or flooding can disturb this water film adhering to the root surface. In addition, anaerobiosis or hypoxia greatly increased Al toxicity and Al uptake by roots probably as a consequence of plasma membrane injury during low oxygen concentration, which can occur strictly in the root tip cells also in well-aerated hydroponics (Wagatsuma 1983).

In our experiments control roots were able to increase pH of solution applied to filter paper from 4 to about 5.6 during 24 h of cultivation, while Al (2 mM) treated roots increased pH to the value of about 4.65. Resulting from these pH shifts, further decrease of free Al concentration occurred. At pH 4.65 only 39 % of total Al is in its free Al^{3+} toxic form (Miyasaka *et al.* 1989). Taken together, the applied 2 mM Al concentration declined after binding of Al^{3+} to filter paper to 220 μM Al, which decreased as a consequence of alkalization to approximately 85 μM Al.

Changes of pH value in the root surrounding causing the decrease of Al concentration were besides Al binding by filter paper next reason for application mM Al range. To induce similar Al toxicity symptoms as were described on barley roots cultivated in hydroponics (Tamás *et al.* 2001, Mistrík *et al.* 2002), 100 times higher Al concentration had to be applied to filter paper.

It is generally known that germination and subsequent roots emerging are less sensitive to Al than established seedlings (De Lima and Copeland 1990). Short-term exposure (24 h) of barley roots to 10 mM Al on filter paper did not induce lethal injuries and evident recovery was observed mainly in Al-tolerant barley cultivar (Tamás *et al.* 2003). Despite the high Al concentration, barley roots grown on filter paper were able to inactivate or exclude Al from root. Especially exudation and accumulation of chelating compound such as organic acids play an important role in resistance of root cells to Al. We showed that Al accumulated only in peripheral region of root tissue stressed by concentrations up to 2 mM was rapidly loosened in recovery period. The accelerated cell turnover on the root surface may help to protect deeper cell layers essential for root growth (Delisle *et al.* 2001). This outer layer probably sufficiently protects roots by chelating Al and pH adjustment, which is disturbed by cultivation in hydroponics causing that plants are more sensitive to Al in solution compared to soil, sand and filter paper cultivation.

Any deviation from optimal physiological conditions may cause an increase in Al accumulation as a consequence of disturbed energy-dependent exclusion mechanism as it was shown in wheat plants (Zhang and Taylor 1991). Villagarcia *et al.* (2001) showed that about 100 times higher Al concentration is required to inhibit root elongation in sand culture in comparison with hydroponics, and that tolerant soybean cultivar in sand culture was remarkably more resistant to Al than in hydroponics. Our experiments screening barley cultivars for Al tolerance revealed no differences between hydroponics and filter paper cultivation system. Barley cv. Bavaria was the most Al tolerant from the analyzed cultivars and cv. Alfor was among the sensitive cultivars both by screening on filter paper or in hydroponics (Tamás *et al.* 2001, 2003).

Application of filter paper for Al toxicity tests allows analyzing the effect of Al directly to caryopses and emerging roots during germination. Its main advantage compared to hydroponics is maintenance of unstirred liquid film around root surface. Our results suggest its crucial role in rapid alteration of surrounding pH in acid environment. The role of root exudates accumulated around the root surface of barley plants cultivated on filter paper and their role in Al toxicity is the subject of ongoing experiments.

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