The role of root exudates in aluminium resistance and silicon-induced amelioration of aluminium toxicity in three varieties of maize (Zea mays L.)

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Received 19 January 2001; Accepted 8 February 2001

Abstract

Aluminium (Al) toxicity is widely considered to be the most important growth-limiting factor for plants in strongly acid soils (pH < 5.0). The inhibition of root elongation in three varieties of maize (Zea mays L. vars Clavito, HS701b and Sikuani) was followed over the first 48 h of Al treatment, and during the initial 10 h elongation was determined on an hourly basis. The silicon (Si)-induced amelioration of Al toxicity was investigated by pre-treating seedlings for 72 h in nutrient solutions with 1000 μM Si before transfer into solutions with 0, 20 or 50 μM Al (without Si). Plants were either grown in complete low ionic strength nutrient solutions (CNS) or in low salt solutions of 0.4 mM CaCl\textsubscript{2} (LSS). In addition, the role of root exudation of organic compounds as a mechanism of Si-induced alleviation of Al toxicity was investigated. Aluminium-induced inhibition of root elongation in the maize var. HS701b was observed within 1 h of Al exposure. After a lag time of at least 8 h, Si-induced alleviation of Al toxicity was observed in this variety when grown in LSS. In the Al-resistant var. Sikuani, Al-resistance was only observed after exposure to 50 μM Al, and not after exposure to 20 μM Al, suggesting that there exists a threshold Al concentration before the mechanisms of Al resistance are activated. Aluminium stimulated root exudation of oxalic acid in all three varieties, but exudate concentrations did not increase with either Al resistance or with Si pretreatment. Aluminium and Si triggered release of catechol and of the flavonoid-type phenolics: catechin, and quercetin. In the Al-resistant variety, Sikuani, Al-exposed plants pretreated with Si exuded up to 15 times more phenolics than those plants not pretreated with Si. The flavonoid-type phenolics, to date unconsidered, appear to play a role in the mechanism(s) of Si-induced amelioration of Al toxicity.

Key words: Aluminium toxicity, catechin, organic acids, phenols, root exudates, silicon, Zea mays L.

Introduction

The physiological basis of mechanisms of aluminium (Al) resistance in plants continues to remain poorly understood, despite current interest in this field. Such resistance should arise from either the plant’s ability to exclude Al from the root or its ability to detoxify Al within the plant (Kochian, 1995; Taylor, 1995). In consideration of recent evidence it appears that organic acids, with Al-chelating abilities, play an important role in the detoxification of Al both externally and internally (Ma, 2000). Organic acid anion secretion has been shown to be Al\textsuperscript{3+} -specific and localized to the root apex (Ma, 2000; Ryan et al., 1995). Aluminium chelation by organic exudates reduces the activity of free Al ions and, consequently, their binding to the root cell wall and/or plasma membrane. The kinds of organic acids secreted by Al-exposed roots vary depending upon the plant species, but secretion of citrate and malate are the most commonly cited. Aluminium-induced secretion of citrate was shown in both maize and snapbean (Miyasaka et al., 1991; Pellet et al., 1995). Likewise, malate is secreted from the roots of Al-resistant cultivars of wheat after Al exposure (Basu et al., 1994; Cocker et al., 1998a; Delhaize et al., 1993). In all

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three species secretion was greater (up to 10-fold) in Al-resistant cultivars than in Al-sensitive ones.

Induced exudation of organic acids has also been proposed as a potential mechanism of the Si-induced amelioration of Al toxicity in higher plants. It has previously been shown that the roots of teosinte treated with Al and Si contained a higher concentration of malate than roots treated with Al only (Barceló et al., 1993). More recently, in their model of Si-induced amelioration of Al toxicity (Cocker et al., 1998b), it was hypothesized that, through Al/Si interactions at the cell wall, the exudation of malate (or other organic compounds) into the cell wall promotes the formation of aluminosilicates (AS) and hydroxyaluminosilicates (HAS), thereby detoxifying Al. In agreement, evidence of Al/Si co-deposition in plant tissues is accumulating (for a review see Hodson and Sangster, 1999). Aluminium/silicon co-deposits were detected in the outer tangential walls of the root epidermis of wheat (Cocker et al., 1997) and inside the vacuoles of maize root cortical cells (Vázquez et al., 1999).

If the formation of Al-organic chelates has an important role in the mechanisms of Al resistance, and/or Si alleviation of Al toxicity, it follows that other oxygen donor compounds with strong Al-binding affinity could play an equally vital role in Al resistance. Several types of phenolics, such as the flavonoid-type phenols, also show strong Al-chelating ability and their exudation by root tips could potentially detoxify Al.

The objectives of this study were to investigate the potential mechanism(s) of Al resistance, and Si-induced Al resistance, through the exudation of organic compounds by the root apices. A comprehensive range of organic acids was analysed in collected root exudates and, in addition, this line of investigation was extended to incorporate phenolics. The exudation of both organic acids and phenolics from the root apices of three varieties of maize (Zea mays L. vars Sikuani, Clavito and HS701b) was determined in plants exposed to Al (0, 20 or 50 μM) previously pretreated with or without Si (1000 μM). The use of Si pretreatment ensures any Si amelioration of Al toxicity observed is not the result of chemical interactions in the bulk solutions, but rather a reflection of interactions within the plant (Cocker et al., 1998b; Corrales et al., 1997). As far as it is known, the potential involvement of the exudation of phenolics by root apices in the mechanisms of both Al resistance and Si-induced amelioration of Al toxicity has not, to date, been considered in this line of research.

Materials and methods

Plant material and growth conditions

Seeds of three varieties of maize (Zea mays L. vars Clavito, HS701b and Sikuani) were surface-sterilized, germinated at 25 °C for 4 d between sheets of filter paper moistened with CaSO₄ (0.5 mM), and grown in cell culture flasks (700 ml capacity) with continuously aerated solutions at pH 4.3. Plants were either grown in complete nutrient solutions (CNS) or in low-salt solutions of 0.4 mM CaCl₂ (LSS). The composition of the CNS was as follows (in mM): 500 Ca(NO₃)₂, 395 K₂SO₄, 5 KH₂PO₄, 100 MgSO₄, 200 NH₄NO₃, 0.06 (NH₄)₆Mo₇O₂₄·5 MnSO₄, 0.38 ZnSO₄, 0.16 CuSO₄·5 H₂O, and 10 FeEDTA. Nutrient solutions were renewed on a daily basis. Half of the plants received the basic nutrient solution (-Si), while the rest were exposed to solution supplemented with 1000 μM Si (+Si). Silicon was added in the form of silicic acid obtained by passing sodium silicate through a H⁺ loaded Dowex 50 W × 8 cation exchange resin (Corrales et al., 1997).

After 72 h in +Si or –Si solutions, solutions were drained off from the culture flasks and renewed with the basic nutrient solution (either CNS or LSS) as above without Si but supplemented with 0, 20 or 50 (only Sikuani) μM Al as AlCl₃·6H₂O. The following abbreviations are used corresponding to the treatments: –Si –Al, –Si +Al, +Si –Al, and +Si +Al. According to the GEOCHEM speciation program (Parker et al., 1987) the free Al³⁺ activities in the solutions were 2.6 and 17.3 μM for solutions with nominal Al concentrations of 20 and 50 μM, respectively.

The seedlings were grown in an environmentally controlled growth chamber under the following conditions: 16/8 h light/darkness, day/night temperature 26/20 °C, RH 70%, and PPFD 190 μmol m⁻² s⁻¹.

Root growth

Cell culture flasks containing plant roots were photographed using OPTIMAS v6.0 at time 0 (before Al treatment), and then after 30 min. 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 24 h, and 48 h of Al treatment. Root elongation was measured by overlaying each photograph in Photoshop v5.1 and measuring the distance (in mm) between root tips at time n and time n + 1. Rates of root elongation (RER, cm h⁻¹) were determined. This system of photographing the flask allows the monitoring of root elongation but avoids the manhandling of the root system.

Growth data are the means of 4–6 plants per treatment and were statistically analysed using a repeated measures analysis of variance design.

Collection of root exudates

For these experiments, seedlings were grown under sterile conditions and all manipulations were carried out in a sterile laminar-flow hood. All solutions were prepared using analytical grade reagents and ultra pure water (Milli-Q plus system, Millipore Corporation, USA). Seeds of the three varieties of maize were surface-sterilized with 5% NaOCl and rinsed in 18 MΩ water. Seeds were placed in autoclaved glass Petri dishes on filter paper moistened with sterile 1 mM CaSO₄. Petri dishes were placed in the dark at 25 °C for 7 d. Seedlings were then transplanted into sterile containers with two compartments. The outer and inner compartments contained 150 ml and 10 ml of filter-sterilized growth solution, respectively (complete nutrient solution or 0.4 mM CaCl₂). The basal nutrient solution was the same composition as described above. Plants used for the determination of phenolic root exudates were grown in complete nutrient solutions, since chlorides interfere with the detection of phenolics. Plants used to determine root exudation of organic acids were grown in solutions of 0.4 mM CaCl₂. The root tips
of two primary roots were secured through two holes in the wall of the inner compartment. Seedlings were pretreated for 72 h in +Si or -Si solutions, and then transferred into solutions with 0, 20 or 50 (only Sikuani) μM Al, as in the growth experiments described above. After 24 h of Al treatment four 5 ml samples of solution were collected and freeze-dried until analysis: two from the inner root-apex compartment and two from the outer mature-root compartment. At the same time, four subsamples (200 μl) of nutrient solution (per container) were incubated for 48 h at 37 °C on agar to check for contamination.

**Analysis of organic acids**

Freeze-dried samples of nutrient solution (2×5 ml) were dissolved in 5 ml of H₂O (18 MΩ) and acidified to pH 3.8 using 0.1 M HCl. Samples were then passed through a cationic resin (AG 50W-X8, Bio-Rad) to remove interfering ions (such as Al³⁺ and Ca²⁺) that could form strong complexes with any organic acids present and prevent their detection in the gas chromatography. Mixtures of solution and resin were filtered through a 0.45 μm filter and filtrates freeze-dried. The freeze-dried samples were redissolved in 0.2% hydroxylationammonium chloride (0.75 ml). Glutaric acid (15 μl of 15 mM concentration) was added as an internal standard. The solution was passed through an anionic resin (Anion AG 1-X8, Bio-Rad) and the supernatant discarded. Organic acids were liberated from the resin into 50% formic acid (0.75 ml). After thorough mixing, the formic acid was passed through a cationic resin (AG 50W-X8, Bio-Rad); an aliquot of 0.5 ml was withdrawn, air-dried and stored until analysis. The following organic acids were detected using a gas chromatograph (Hewlett Packard 5890, serie II) equipped with a flame ionization detector and a capillary column 77710 (Chrompack, Middleburg, The Netherlands): acetic acid, citric acid, fumaric acid, maleic acid, malic acid, malonic acid, oxalic acid, succinic acid, and tartaric acid.

**Analysis of phenolic compounds**

Extraction of soluble phenolics was performed as described earlier (Solecka et al., 1999). Lyophilized samples of nutrient solutions were redissolved in 5 ml H₂O MQ and extracted three times with ethyl ether. The water fractions (containing soluble phenolics) were hydrolyzed under alkaline conditions (pH 13, 4 h, N₂, 25 °C). After hydrolysis, samples were acidified to pH 3 and extracted three times with ethyl acetate. The organic fraction was evaporated to dryness and redissolved in MeOH: H₂O (1:1 v/v).

The phenolic fractions were identified by an HPLC procedure on a Shimadzu system (System Controller SCL-10ADvp) by means of a C18 Nucleosil column (4 mm–25 cm, Scharlau SA, Barcelona) and two solvents: solvent A: solvent A was composed of 2% acetic acid (v/v, in water) and solvent B acetonitrile-water-acetic acid (8:2:0.2, by vol.), with a linear gradient from 4% to 90%. Phenolic compounds were monitored and analysed with a diode-array detector (250–400 nm, Shimadzu SPD-M10A priests) and compared with external standards (prepared in MeOH: H₂O (1:1 v/v)): Apigenin, (+)-catechin, catechol, chlorogenic acid, p-coumaric, curcumin, 2,3-dimethoxybenzaldehyde, 3,5-dimethoxy-4-hydroxycinnamic acid, (+)-epicatechin, ferulic acid, gallic acid, 2′-hydroxy-4′, 6′-dimethoxycatephenone, (±)-methoxyphenylacetic acid, (±)-naringenin, protocatechuic acid, quercetin, (±)-taxifolin, and vanillic acid from Sigma.

**Results**

**Root elongation in Zea mays L. var. HS701b**

Aluminium induced an immediate and significant reduction in root elongation rates (RER), and significant differences in RER between Al-treated plants (-Si + Al) and controls (-Si – Al) were observed after only 30 min or 1 h in LSS (P ≤ 0.01) and CNS (P ≤ 0.001), respectively (Fig. 1; Table 1). In LSS, Al-induced inhibition of RER increased with time: after 4, 10 and 48 h of Al exposure, root growth was 44%, 27% and 12% of controls, respectively.

In CNS, pretreatment with Si had no alleviating effect on the Al-induced inhibition of root elongation (Figs 1, 2). In contrast, in LSS, pretreatment with Si significantly improved RER in Al-treated plants (P ≤ 0.05). At harvest RER of Al-treated plants pretreated with Si were 55% of the corresponding controls compared with 12% when not pretreated with Si (Fig. 2d). Silicon-induced amelioration of Al-inhibition of root elongation began after 8 h of Al treatment: between 8 and 48 h RER was significantly greater in Al-treated plants pretreated with Si than in those not pretreated (Fig. 1). Amelioration did not decrease with Al-exposure time: root growth in +Si + Al plants was about 55% of controls throughout the 48 h of Al treatment.

**Root elongation in Zea mays L. var. Clavito**

Aluminium-induced inhibition of root growth was only visible after at least 8 h of Al-treatment, (Fig. 3; Table 1) irrespective of the growth medium (CNS or LSS). In CNS, during the initial 3 h of Al exposure RER was significantly greater in Al-treated plants (-Si + Al) than in controls (-Si – Al). Although less extreme than observed in the var. HS701b, between 8 and 48 h of treatment, Al significantly reduced root elongation, and after 48 h root growth was 68% and 69% of that of control plants in CNS and LSS, respectively (Fig. 4). Pretreatment with Si had no alleviating effect, and this was observed whether growth had been in a CNS or a LSS.

**Root elongation in Zea mays L. var. Sikuani**

Figures 5–8 (and Table 1) show the RER with time in the maize var. Sikuani pretreated with or without Si and then transferred into solutions with Al at either 20 μM or 50 μM. In CNS, Al at 20 μM significantly reduced root elongation (P ≤ 0.001); at harvest RER of Al-treated plants (-Si + Al) was 46% that of control plants (Fig. 6). Aluminium-induced inhibition of root growth started after 8 h of exposure (Fig. 5). Silicon pretreatment had no alleviating effect (Figs 5, 6). In LSS, Al had an inhibiting effect on root growth but only after 10 h of treatment: root elongation of Al-exposed plants (-Si + Al) was 27% of controls (-Si – Al) after 48 h (Fig. 6d). However, in this
growth medium Si significantly improved root growth with time, and at harvest root growth of +Si + Al plants was increased to 58% of control plants; the ameliorative effect of Si was time-dependent (P ≤ 0.001).

After exposure to a higher concentration of Al (50 μM) the Sikuani variety showed Al resistance. Aluminium significantly reduced root elongation almost immediately (within 30 min of exposure; Fig. 7), however, after 4 h of exposure RER recovered and there were no further significant differences between the RER of control plants (–Si – Al) and those of Al-treated plants (–Si + Al; Figs 7, 8). This was particularly evident in plants grown in CNS.

Effect of growth medium (CNS versus LSS)

The performance of the three varieties differed significantly between CNS and LSS (to at least P ≤ 0.05). In LSS compared to CNS, RER were up to 2-fold faster (throughout the 48 h of treatment) in both the var Clavito and Sikuani, and particularly in control treatments. Aluminium-induced reductions in RER were less extreme in LSS in both the var Clavito and Sikuani (at 20 μM Al). The beneficial effects of Al on RER in Clavito, during the initial 3 h of Al exposure, were not observed in LSS. Si-induced amelioration of Al toxicity in HS701b and in Sikuani (at 20 μM Al) was only observed in LSS.

Fig. 1. Root elongation (cm, ± SE) of Zea mays L. var. HS701b exposed to 0 or 20 μM Al after pretreatment with or without 1000 μM Si. Plants were grown in complete nutrient solutions (CNS; A, B) or in CaCl₂ solutions (LSS; C, D). Root elongation is shown over the first 10 h of Al treatment.

Table 1. A summary of the growth-response of the three maize varieties (Zea mays L. vars HS701b, Clavito and Sikuani) to Al exposure with or without Si pretreatment

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>RER Inhibited (20 μM Al)</th>
<th>RER Inhibited (50 μM Al)</th>
<th>Time-lag for inhibition</th>
<th>Si-induced alleviation</th>
<th>Time-lag for alleviation</th>
<th>Differences between LSS and CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS701b</td>
<td>Yes</td>
<td>–</td>
<td>&lt;1 h</td>
<td>Yes</td>
<td>8 h</td>
<td>Yes</td>
</tr>
<tr>
<td>Clavito</td>
<td>Yes</td>
<td>–</td>
<td>8 h</td>
<td>No</td>
<td>–</td>
<td>Yes</td>
</tr>
<tr>
<td>Sikuani</td>
<td>Yes</td>
<td>No</td>
<td>8–10 h</td>
<td>Yes (LSS, 20 μM Al)</td>
<td>&gt;10 h</td>
<td>Yes</td>
</tr>
</tbody>
</table>

LSS, low salt solution; CNS, complete nutrient solution.
Exudation of organic acids by maize seedlings

The exudation of organic acids from the root tip was determined over 24 h of exposure to 20 μM Al (all three cultivars) or 50 μM Al (Sikuani) and results are expressed as nmol h⁻¹ tip⁻¹ (Fig. 9; Table 2). Of all the organic acids detected only three showed significant differences between either treatments or varieties: acetic acid, citric acid and oxalic acid. Seedlings grown in control nutrient solutions (−Si –Al) exuded small amounts of oxalate (about 0.5 nmol h⁻¹ tip⁻¹). Aluminium (at either 20 or 50 μM) stimulated oxalate secretion, and concentrations increased by up to 17-fold. Oxalate secretion did not increase with increasing Al concentration (Sikuani). There was no significant difference in the concentration of oxalate exuded from Al-treated root tips pretreated with Si (+Si +Al) and those not pretreated with Si (−Si +Al). In the presence of Al, Si pretreatment stimulated the exudation of aconitate in Clavito and citrate in HS701b. However, concentrations of these two acids were at least 10-fold less than those of oxalate.

Exudation of phenolics by maize seedlings

The exudation of phenolics from the root tip was determined over 24 h of exposure to 20 μM Al (all three cultivars) or 50 μM Al (Sikuani) and results are expressed as nmol h⁻¹ tip⁻¹ (Fig. 10; Table 2). Four main phenolics were detectable: catechin, catechol, curcumin, and quercetin. Secretion of catechin, catechol or quercetin in seedlings grown in control nutrient solutions (−Si −Al) was either not detected or detected at low concentrations (about 2.5 nmol h⁻¹ tip⁻¹). Aluminium exposure (and Si pretreatment without subsequent Al exposure) stimulated exudation of phenolics. However, significant differences in exudation between varieties and treatments were observed. In HS701b, Al exposure enhanced exudation of catechol and curcumin, but differences in the concentration exuded between Al-exposed plants pretreated with Si and those not Si pretreated were small. In the same variety, Si pretreatment induced quercetin exudation whether plants were exposed to Al or not. Catechin was not observed in the exudates of this Al sensitive
Fig. 3. Root elongation (cm, \( \pm \) SE) of *Zea mays* L. var. Clavito exposed to 0 or 20 \( \mu \)M Al after pretreatment with or without 1000 \( \mu \)M Si. Plants were grown in complete nutrient solutions (CNS; A, B) or in CaCl$_2$ solutions (LSS; C, D). Root elongation is shown over the first 10 h of Al treatment.

Variety. In Clavito, both Si and Al treatments enhanced exudation of curcumin, while Si alone or in combination with Al caused catechin exudation. Silicon pretreatment, but not after subsequent exposure to Al, enhanced catechin exudation. In contrast, Si-pretreatment induced root tip exudation of both catechin and quercetin after Al exposure in the Sikuan variety, and this stimulation significantly increased (up to 15-fold) with increasing Al concentration from 20 to 50 \( \mu \)M.

### Discussion

The Al-sensitivity of the maize var. HS701b has been shown in previous investigations both in nutrient solutions and in the field (Günsé et al., 2000; Vázquez et al., 1999). The results presented here confirm that this variety is Al-sensitive. The maize var. Clavito has previously been shown in the field to be of intermediate acid soil tolerance, while the variety Sikuan exhibits high tolerance to acid soil conditions (LA Rojas, Final Report EC project ERBIC 18 CT 96 0063, 2000). The results of the current hydroponic study found that the variety Clavito showed a certain degree of Al resistance. Aluminium resistance in the var. Sikuan was only observed when exposed to 50 \( \mu \)M Al. A lack of consistency between solution culture experiments and field studies has often been cited (Horst, 1995). Part of the problem may arise from the difficulty of isolating Al toxicity from the additional adverse conditions experienced in the field. However, the use of nutrient solutions that poorly reflect soil solutions from acidic sites may also account for discrepancies between field and solution studies.

Many Al-toxicity studies are conducted in low-salt basal solutions, such as CaCl$_2$ or Ca(NO$_3$)$_2$, although it seems unlikely that these background solutions should better simulate field conditions than complete nutrient solutions of low ionic strength. In agreement with others (Lazof and Holland, 1999), it was found that the growth response to Al/Si of the three maize varieties was dependent upon the type of basal solution: CNS or LSS. Aluminium inhibition of RER in HS701b increased with
time only in LSS, but conversely Al inhibition of RER in Sikuani and Clavito was less extreme in LSS. Furthermore, Si-induced amelioration of Al-toxicity (in all three varieties) was only observed in LSS. Growth-enhancing effects of Al in legumes were not observed in LSS (Lazof and Holland, 1999), and in agreement, the initial beneficial effect of Al in the maize var. Clavito was observed only in CNS. These results show that it cannot be assumed that genotypes will perform similarly whether grown in complete nutrient solution or low-salt solutions. In addition, the differences in the growth response suggest that different mechanisms of Al inhibition of RER, and Si-induced amelioration of Al toxicity, are operating in these two basal solutions.

In the Al-sensitive maize var. HS 7777 an inhibition of root elongation was observed after less than 30 min of exposure to Al (20 μM Al; Llugany et al., 1995). In agreement, this study has shown a rapid Al-induced reduction in RER (after at most 30 min) in the Al-sensitive maize variety HS701b. In the var. Clavito, which showed a certain degree of Al-resistance, and in the Al-resistant variety Sikuani when exposed to 20 μM Al in CNS, Al-induced reductions in RER were not observed until after at least 8 h of exposure.

It was shown that Al (at 20 μM) caused a significant decrease in RER in an Al-resistant maize var. C525 M after 112 min of exposure, whereas after 24 h RER did not differ from that of controls (Llugany et al., 1995). In a separate study, ultrastructural changes in this variety of maize, such as cell wall thickening, observed during the initial 4 h of Al treatment were not seen after 24 h exposure (Vázquez et al., 1999). More recently, it was observed that Al resistance in the resistant maize var. ATP SR Yellow showed a lag time of at least 4 h to come into effect (Gunsé et al., 2000). In line, callose formation, a strong marker of Al sensitivity in maize (Horst et al., 1997), was high after 4 h exposure but not after 24 h. In the present investigation the Al-resistant var. Sikuani, when exposed to 50 μM Al, showed a similar lag time of at least 3 h before Al resistance was observed, and
between 4 and 48 h RER did not differ from that of the controls. This was most evident in plants grown in complete nutrient solutions of low ionic strength and less so when a background solution of CaCl$_2$ was used. This result corroborates the findings of the above investigation using the var. ATP SR Yellow and suggests that in these maize varieties the Al resistance mechanism is not active in the absence of Al, but requires induction upon exposure. The observation that Sikuniu suffered inhibition of RER by 20 μM Al, but not when exposed to 50 μM, indicates that a minimum Al concentration (or threshold) may be required before the mechanisms of Al resistance are activated. The growth-response of this variety is not in line with the three classic dose–response curves for potentially toxic substances (Ernst, 1998): (a) a negative linear relationship with no non-toxic concentration; (b) a hyperbolic relationship where resistance exists up to a threshold concentration at which point toxicity occurs; and (c) a hormesis-type relationship where growth stimulation occurs at low concentrations of the potentially toxic substance. The results observed here suggest that a further type of dose–response curve exists: a threshold metal concentration for resistance.

This study has shown significant differences in the growth response of three maize varieties to Al in relation to the duration of Al-exposure. The Si-induced amelioration of Al toxicity, observed in the var HS701b (in LSS) and Sikuniu (in LSS at 20 μM), were also time-dependent (Al × Si × Time interaction factors were significant at $P \leq 0.001$). In the Al-sensitive var. HS701b, and in the var. Clavito (although differences were not found to be significant in this variety during the initial 10 h), a lag time of at least 8 h Al exposure was required before Si-induced alleviation of Al-inhibited root elongation was observed. As far as is known, this is the first time that chronological changes in Si-induced amelioration of Al toxicity have been observed. In the var. Clavito this alleviating effect was only transient, and after 48 h of exposure RER of +Si + Al plants was no greater than −Si + Al plants. This decrease of Si-induced alleviation of Al toxicity with time was probably caused by a decrease of tissue Si concentrations due to growth. In the var. Sikuniu significant Al-induced inhibition of RER (at 20 μM Al) was observed between 24 and 48 h, and Si-induced alleviation occurred during the same time period. The exposure of plants to Si prior to Al treatment in

\[ \text{Fig. 5. Root elongation (cm, ± SE) of Zea mays L. var. Sikuniu exposed to 0 or 20 μM Al after pretreatment with or without 1000 μM Si. Plants were grown in complete nutrient solutions (CNS; A, B) or in CaCl}_2\text{ solutions (LSS; C, D). Root elongation is shown over the first 10 h of Al treatment.} \]
Fig. 6. Root elongation (cm, ± SE) of Zea mays L. var. Sikuan exposed to 0 or 20 mM Al after pretreatment with or without 1000 mM Si. Plants were grown in complete nutrient solutions (CNS; A, B) or in CaCl₂ solutions (LSS; C, D). Root elongation is shown after 24 h (A, C) and 48 h (B, D) of Al treatment.

Aluminium-induced enhancement of organic acid anion exudation has been proposed as a means of Al detoxification in Al-resistant varieties of different plant species (Ma, 2000). Most experimental evidence comes from investigations in wheat, where malate exudation from root tips seems to be an important mechanism for Al detoxification (Delhaize et al., 1993). However, even in this species multiple aluminium exclusion mechanisms seem to be implied in Al resistance (Pellet et al., 1997). The few investigations addressing organic acid anion exudation in response to Al in maize varieties differing in Al resistance reported higher exudation of citrate in resistant varieties than in sensitive ones (Pellet et al., 1995; Jorge and Arruda, 1997). Less varietal differences in malate exudation were observed and no oxalate, succinate or isocitrate was detected in the exudates (Jorge and Arruda, 1997). This clearly contrasts with these experiments where oxalate was the most abundant organic acid in exudates of Al-exposed root tips (Fig. 9). Aluminium-induced citrate exudation was only observed in Si pretreated plants of the Al-sensitive variety HS701b and in Clavito, the variety with intermediate Al sensitivity. It is, however, doubtful if the protective effect of the Si treatment can be attributed to citrate exudation by the root tips. Oxalate exudation in response to Al occurred in all varieties regardless of the Si supply. Differences in exudate composition between the varieties used in this study and those analysed by other authors could be due to varietal differences but could also be the result of the different analytical methods employed by the various authors. For example, the GC methods employed here are likely to detect lower concentrations of organic acids than the commonly used enzyme kits (Delhaize et al., 1993; Jorge and Arruda, 1997). In this study, two sets of
Fig. 7. Root elongation (cm, ± SE) of Zea mays L. var. Sikuani exposed to 0 or 50 μM Al after pretreatment with or without 1000 μM Si. Plants were grown in complete nutrient solutions (CNS; A, B) or in CaCl₂ solutions (LSS; C, D). Root elongation is shown over the first 10 h of Al treatment.

Standards were prepared: (1) the complete set of organic acids with 0.4 mM Ca and 20 μM (or 50 μM) Al; and (2) the complete set of organic acids with 0.4 mM Ca. Standards and samples underwent exactly the same procedure before injection into the GC. During an initial step, samples (and standards) were acidified to pH 3.8 and passed through a cationic resin, the quantity of which was calculated so as to remove all Al³⁺ and Ca²⁺ ions. In this manner, the amount of organic acid anions that go undetected by the GC due to the formation of Al-organic complexes can be limited.

The Al-detoxifying capacity of organic acid anions is related to their structural configuration. Effective detoxifiers fall into two categories: those with two pairs of OH/COOH attached to two adjacent carbons or those with two COOHs directly connected (Hue et al., 1986). Oxalic acid falls into the second category and forms stable 5-bond ring structures with Al. The capacity for oxalate to detoxify Al is considered to be lower than that of citrate, but higher than that of malate (the log Ks for the stability constants: Al-citrate (12.26) > Al-oxalate (6.53) > Al-malate (6.00)). Oxalate has also been found in the root exudates of buckwheat and taro in response to Al (Ma et al., 1997; Ma, 2000). This is the first study to show Al-induced secretion of oxalic acid from maize root tips. However an Al-induced enhancement of oxalate exudation was observed in all three varieties, meaning that exudation of oxalate may not be responsible for varietal differences in Al resistance. The synthesis and intracellular accumulation of oxalate in plants is a common phenomenon and has been implicated in cell Ca homeostasis (Ilarslan et al., 1997; Webb, 1999).

Nonetheless, Al resistance in the maize variety Sikuani of the present study, as in other Al-resistant maize varieties such as C 525 M (Vázquez et al., 1999) or ATP SR Yellow (Gunsé et al., 2000), must be related to some kind of apoplastic detoxification of Al. This is clearly indicated by the fact that the phenotypic expression of resistance in the form of a recovery of the root elongation rate was coincident in time with a decrease in stainability of apoplastic Al by either morin (unpublished data) or hematoxylin (Jorge and Arruda, 1997; Gunsé et al., 2000).

According to the results (Fig. 10) Al-induced enhancement of exudation of phenolics may play an important
Fig. 8. Root elongation (cm, ± SE) of Zea mays L. var. Sikuan exposed to 0 or 50 μM Al after pretreatment with or without 1000 μM Si. Plants were grown in complete nutrient solutions (CNS; A, B) or in CaCl₂ solutions (LSS; C, D). Root elongation is shown after 24 h (A, C) and 48 h (B, D) of Al treatment.

Table 2. A summary of the root exudation characteristics of the three maize varieties (Zea mays L. var HS701b, Clavito and Sikuan) to Al exposure with or without Si pretreatment

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Trends observed in the exudation of organic anions</th>
<th>Trends observed in the exudation of phenolics</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS701b</td>
<td>Al stimulated 17-fold increase in oxalate. Al after Si pretreatment stimulated exudation of citrate but not oxalate</td>
<td>Al stimulated CAT (160-fold) and CUR (2-fold) exudation. No effect of Si pre-treatment prior to Al exposure</td>
</tr>
<tr>
<td>Clavito</td>
<td>Al stimulated 17-fold increase in oxalate. Al after Si pretreatment stimulated exudation of aconitate induced secretion but not oxalate</td>
<td>Al stimulated CUR (17-fold) exudation. Si pretreatment prior to Al exposure induced secretion of CAT</td>
</tr>
<tr>
<td>Sikuan</td>
<td>Al stimulated 15-fold increase in oxalate. No effect of Si-pretreatment</td>
<td>Al stimulated CAT (2-fold), CATE (225-fold), QUE (2-fold), and CUR (24-fold) exudation. Si pretreatment prior to Al exposure led to up to 15-fold increase in exudation</td>
</tr>
</tbody>
</table>

role in the detoxification of Al in the root tip apoplast. The most notable phenolics detected at substantial concentrations in exudates of the experimental plants used here were catechol and the flavonoid-type phenolics (catechin and quercetin). Curcumin was also detected, and was found at significantly higher concentrations in -Si + Al and + Si + Al groups than in controls, but its structural configuration suggests they are unlikely to be potential Al chelators. In the varieties HS701b and Clavito significant increases were observed in root exudation of quercetin and catechol, respectively, in Al-treated plants pretreated with 1000 μM Si compared with those not Si pretreated. Similar concentrations of these two phenolics were observed in corresponding plants pretreated with Si but not later exposed to Al (+Si – Al). Much higher concentrations of flavonoid-type phenolics, however, were found in the Al-resistant variety Sikuan in those Al treatments that did not cause inhibition of root growth.
Fig. 9. Root exudation of oxalate, aconitate and citrate (nmol h\(^{-1}\) tip\(^{-1}\), ± SE) from roots of (a) Sikuani, (b) Clavito and (c) HS701b varieties. Plants, pretreated with or without 1000 \(\mu\)M Si, were grown in CaCl\(_2\) nutrient solutions (LSS) with Al (0–20 \(\mu\)M) for 24 h.

(50 \(\mu\)M Al alone or with Si pretreatment, and 20 \(\mu\)M Al with Si pretreatment). In contrast, exposure to 20 \(\mu\)M Al without Si pretreatment did not enhance exudation of flavonoid-type phenolics and inhibited RER. The exudation of flavonoid-type phenolics increased with increasing Al concentration: total exuded phenolics were about 10-fold higher at 50 \(\mu\)M than 20 \(\mu\)M Al.

Aluminium forms stable complexes with some phenolic substances. Stability constants for the complex formation of Al with phenolics can be even higher than for Al-organic acid complexes (Martell and Motekaitis, 1989). However, it has been argued that phenolics may be less important in Al detoxification than organic acids because of the fact that at acid pH there is a strong competition from H\(^+\) in complex formation. This may be the case with simple phenolics such as catechol in soil solutions under acid field conditions (Martell and Motekaitis, 1989). In contrast, there is convincing direct experimental evidence that certain flavonoid-type phenolics readily form complexes with Al in the root apoplast. Morin
only much higher than that for Al complexes with organic acids (see above) but also much higher than the log \( K \) for the proton-catechin complexes (Smith and Martell, 1989). Interestingly, catechin is an abundant component of green tea (Wörth et al., 2000), a species that is known to accumulate and tolerate high tissue levels of Al. Since, in these investigations, both Al-induced enhancement of quercetin and catechin exudation showed good coincidence with the maintenance or recovery of the root elongation rates in the Al-resistant Sikuani, and the exudation rates of these flavonoid-type phenolics were substantially higher than that of organic acids, it is proposed that these flavonoid-type phenolics may play an important role in the apoplastic detoxification of Al in maize root tips.

One of the arguments given against the secretion of organic acid anions as a resistance mechanism of Al toxicity is that the amount of acid secreted by the root tip is insufficient to detoxify all the Al present (Ma, 2000; Pellet et al., 1995). It was found that roots of the Al-exposed plants (Si + Al) exuded about 20-fold more phenolics (total phenolics exuded) than organic acid anions, and that Al-exposed plants pretreated with Si (+Si + Al) could exude up to 150-fold more phenolics than organic acid anions. These results add even further weight to the argument that phenols are likely Al detoxifiers in maize.

It was concluded that Si-induced exudation of flavonoid-type phenolics, especially catechin, by the root apex could be a potential mechanism in the amelioration of Al toxicity by Si, and that this exudation is Al-concentration-dependent. The potential role of phenolics in Al resistance, and the Si-induced exudation of phenolics, is currently under further investigation in this laboratory. The preliminary data reported here are the first to show evidence of a second type of Al-chelator (flavonoid-type phenols) which may play an even more important role in Al detoxification than the commonly investigated organic acids (malate or citrate). Future work in this laboratory will focus on the confirmation of which types of phenolics (using HPLC-MS techniques) are involved in Al resistance, and whether or not mechanisms underlying Si-induced alleviation of Al toxicity involve phenol exudation. Further work will also address the possible role of flavonoid-type phenolics in Al detoxification inside plant cells.

**Acknowledgements**

Supported by the EC (ICA4-CT2000-30017), Spanish Government (DGICYT PB97-0163-C02-01) and, in part, by The Royal Society (London) and the European Science Foundation (Plant Adaptation Programme). The authors thank Isabel Corrales for her help with the sterile-culture technique, and Mrs Rosa Padilla for technical assistance.
References


