Pergamon

PII: S0031-9422(97)00044-7

Phytochemistry, Vol. 45, No. 4, pp. 675–681, 1997

0031-9422/97 $17.00+0.00

ALUMINUM-INDUCED ORGANIC ACIDS EXUDATION BY ROOTS OF AN ALUMINUM-TOLERANT TROPICAL MAIZE

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(*Received in revised form* 18 *November* 1996)

Key Word Index—*Zea mays* L.; maize; Gramineae; aluminum tolerance; organic acids; aluminum exclusion.

Abstract—Aluminum (Al) tolerant and sensitive plants selected from the tropical maize variety Taiúba were grown in complete nutrient and simple salt solutions in the presence and absence of phytotoxic concentrations of Al. During the first 20 hr of Al exposure, the root growth rate of both tolerant and sensitive plants was severely inhibited as a consequence of Al infiltration into the root tip cells. After this period, however, roots of Al-treated tolerant plants recovered to a growth rate similar to that of control plants, while the root growth rate of sensitive plants remained severely inhibited. The recovery of the root growth rate of tolerant plants coincided with the extrusion of the Al that had been absorbed in the first 20 hr of Al exposure. When the roots of tolerant and sensitive plants were grown in simple salt solutions containing a series of Al concentrations, a dose-dependent citrate and malate exudation was observed from tolerant but not from sensitive roots. The level of citrate exudation was two- to four-fold that observed for malate. The organic acid exudation was not influenced by the level of phosphate in the growth solution, suggesting a specific Al-inducing process involved in the Al tolerance in maize. We concluded from these results that the Al infiltration in the roots at the beginning of Al exposure induces the exudation of organic acids which may exclude the toxic ion from the root tip cells of tolerant plants.

INTRODUCTION

Aluminum (Al) is one of the most important toxic elements in acid soils where the low pH increases the Al-solubilization so affecting crop productivity [1]. Toxic levels of Al inhibit the root elongation as a consequence of root apex disruption [2–4].

Al-tolerant plants have been identified in several plant species but the mechanism by which tolerant plants resist phytotoxic concentrations of Al remains obscure. Cellular Al exclusion from the root apex cells have been observed in Al-tolerant plants of wheat [5–7], snap bean [8] and maize [9]. Al-exclusion is the mechanism by which tolerant plants prevent Al penetration by either the exudation of Al-chelating compounds, formation in the rhizosphere of a plant-induced pH barrier, or al immobilization in the cell wall [4, 10].

Exudation of organic acids has been shown to be an important mechanism by which tolerant plants exclude Al from the root tip cells [6–9]. Al-tolerant snap bean roots secrete 10-fold more citrate than an Al-sensitive genotype [8]. However, citrate could be excreted in response to nutrient deficiency. Some studies have demonstrated that citrate and malate exudation is due to Pi deficiency [11, 12], while others demonstrated that malate exudation is stimulated by Al and not by the presence of La, or the absence of Fe or Pi [6].

The kind of organic acid secreted by roots varies from one species to another. Certainly, this should affect the degree of Al tolerance as the constant of association of Al-organic acid varies form one compound to another. Tolerant snap bean secretes citrate [8], whereas tolerant wheat secretes 10-fold more malate and three- to five-fold more succinate than the Al-sensitive seedlings [6]. Tolerant maize secretes citrate and malate, but the amount of citrate is higher than malate, and sensitive plants do not secrete malate but do secrete a small amount of citrate [9]. Maize also excretes Pi in response to Al treatment, which by itself could be an additional important mechanism of Al tolerance in plants [9].

Here we describe a process of Al-induced citrate and malate exudation from the root system from tolerant

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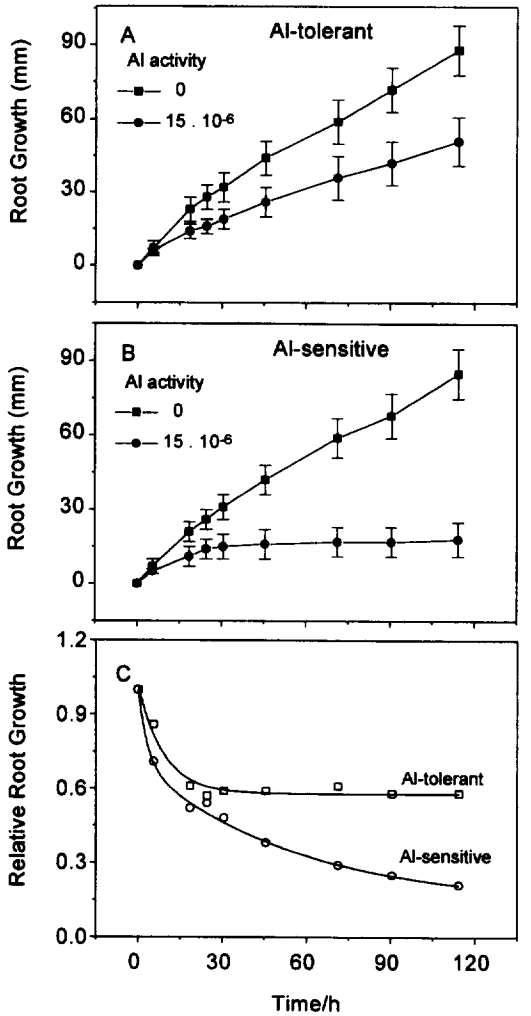

Fig. 1. Effect of Al^{3+} on root development of tolerant and sensitive plants from maize variety Taiúba. Plants were grown in the complete nutrient solution in the absence (■) and presence (●) of a free Al^{3+} activity of 15.2×10^{-6} for 120 hr (A) Al-tolerant; (B) Al-sensitive: (C) RRG of Al-tolerant (□) and Al-sensitive (○) plants. Vertical bars represent ± S.E. ($n = 8$).

plants selected from a tropical maize variety. These two organic acids are induced in a dose-dependent manner in a process which did not depend on the concentration of phosphate in the medium.

RESULTS AND DISCUSSION

Effect of Al, La *and* Ga *on root development of tolerant and sensitive plants*

The root growth rate of tolerant and sensitive plants on complete nutrient solutions with or without an Al^{3+} activity of 15×10^{-6} is shown in Fig. 1. The primary roots of tolerant plants exhibited continuous growth in the presence of Al for a period of 114 hr although the growth rate was lower than that observed for plants developed in the absence of Al (Fig. 1(A)). In contrast, the roots of sensitive plants grew only for the first 24–30 hr and then almost completely stopped development (Fig. 1(B)).

The effect of Al on root growth rate can be demonstrated clearly by the relative root growth (RRG). During the first 20 hr the RRG of tolerant plants reduced to ~60% of the initial values (Fig. 1(C)). The tolerant plants, then minimized the Al toxicity and established a growth rate that was maintained during the period monitored. This suggests that Al toxicity affected root development of tolerant plants only at the beginning of Al treatment. For the sensitive plants, however, the RRG was continuously reduced, indicating that their roots are not able to alleviate the Al toxicity, which drastically affected the root development (Fig. 1(C)).

The inhibition of root growth at the beginning of exposure to Al suggests that during this period Al infiltrates the root tip cells of both genotypes. Staining of Al-treated roots with hematoxylin [13] has previously been used to assess tolerance in barley [14] and to visualize the level of Al infiltration into sensitive and tolerant roots in wheat [6, 15, 16]. Hematoxylin staining was used to monitor the Al infiltration in the root apex of the tolerant and sensitive plants of maize variety Taiúba (Fig. 2). Plants were incubated in complete nutrient solution in the presence of Al, and samples collected at 0, 24 and 72 hr of treatment. After 24 hr of Al exposure, root tips of both tolerant and sensitive plants stained dark blue with hematoxylin indicating that Al penetrated the root apex cells of both genotypes (Fig. 2, column 2). After 72 hr, however, only the root tips of sensitive plants still stained with hematoxylin (Fig. 2, column 3). Indeed, after 36 hr, root tips of tolerant plants recovered the typical pale yellow colour (data not shown). This could be due to a process of extrusion of Al that had been absorbed by tolerant plants in the first 24 hr of treatment. Alternatively, the decrease in staining intensity could be due to a decrease in the formation of Al-hematoxylin complexes over time [16]. However, the fact that the decrease in hematoxylin staining coincided with the recovery of the root growth rate (Fig. 1), clearly suggest that Al is being inactivated in the roots of the tolerant plants.

Lanthanum (La) also affected the root growth rate of tolerant and sensitive plants (Fig. 3). The RRG of tolerant and sensitive plants decreased *ca* 70 (Fig. 3(A)) and 30% (Fig. 3(B)) of the initial values, respectively, when 20 μM of La were added to the complete nutrient solution. Surprisingly, gallium (Ga), which is a trivalent cation of the same family of Al and therefore presents similar chemical properties, stimulated the RRG of both tolerant and sensitive plants at 150 μM (Fig. 3(B)). This is the first report showing a stimulatory effect of a trivalent cation on root development. Delhaize *et al.* [5] observed that Al-tolerant and Al-sensitive wheat plants were equally inhibited by La. However, neither La nor Ga stimulated organic acid excudation from the Al-tolerant wheat plants [6, 17].

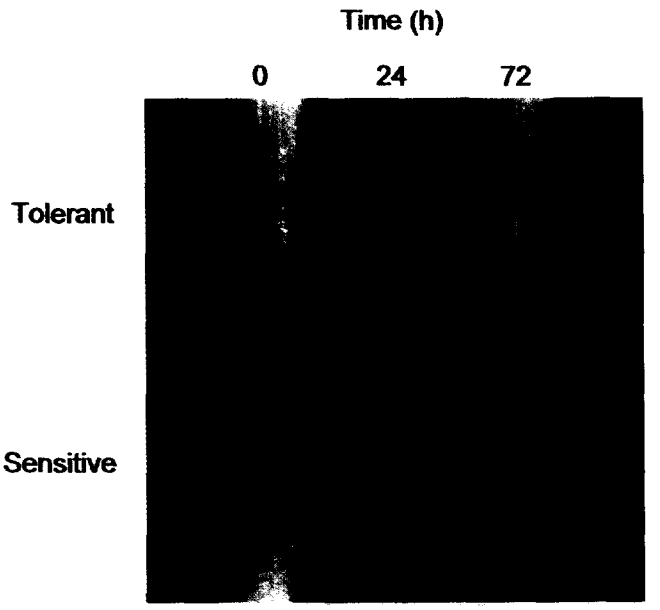

Fig. 2. Hematoxylin staining of root tips of Al-tolerant and -sensitive plants exposed to 36 μM $AlK(SO_4)_2$ for 0, 24 and 72 hr under the same conditions as described in Fig. 1. The dark blue coloration represents the reaction of Al^{3+} with the dye. After 24 hr exposure to Al^{3+} the root tips of sensitive plants were severely damaged.

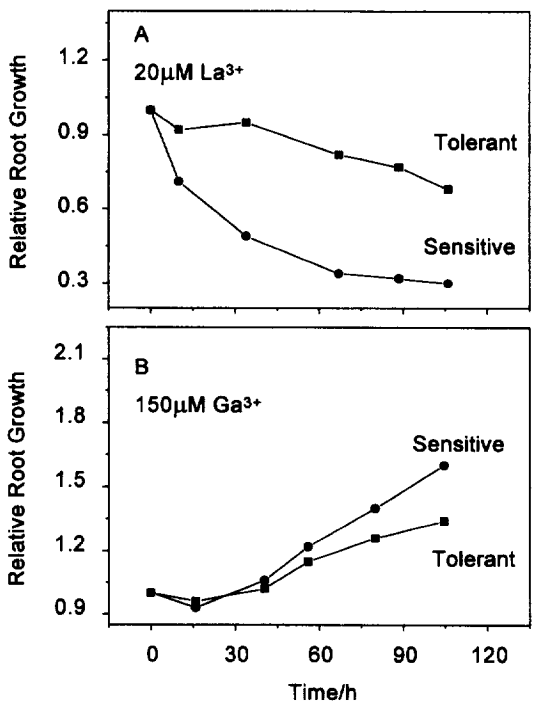

Fig. 3. Effect of trivalent cations on root development of tolerant and sensitive plants from maize variety Taiúba. Plants were grown in the complete nutrient solution in the presence and absence of cations for 120 hr. (A) RRG of Al-tolerant and -sensitive plants growing in the presence of La^{3+}; (B) RRG of Al-tolerant and -sensitive plants growing in the presence of Ga^{3+}.

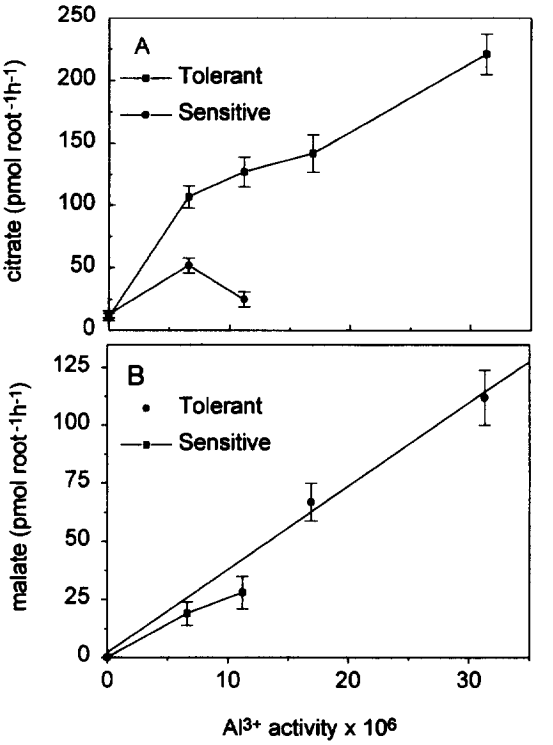

Fig. 4. Citrate and malate exudation from roots of Al-tolerant and -sensitive plants exposed to increasing concentrations of Al^{3+} in the simple salt solution. Exposure time was 30 hr. Each point represents the average of two to three measurements. At Al^{3+} activities above 11.2×10^{-6} the roots of sensitive plants were severely damaged and organic acids must be liberated from the cytoplasm. For this reason the data were not included. Malate and citrate were assayed using enzymic methods and UV spectroscopy.

Exudation of organic acids by maize roots

The hematoxylin staining shown in Fig. 2 confirms that Al infiltrates to a greater extent into roots of Al-sensitive plants than into roots of Al-resistant plants, which is consistent with the hypothesis that Al tolerance is associated with a process of efficient Al exclusion. Exudation of organic acids has been observed in different plant species [5, 6, 8, 9, 17], but are the Al-induced organic acid exudations applicable to all plant species and varieties and to what extent is this phenomenon solely responsible for Al tolerance?

To determine if Al exclusion from roots of the maize variety Taiúba was mediated by exudation of organic acid, tolerant and sensitive plants were exposed to a range concentrations of Al for 30 hr (Fig. 4). The tolerant plants excreted citrate and malate from their roots in a dose-dependent manner. The amounts of citrate excreted (Fig. 4(A)) was two- to four-fold that observed for malate (Fig. 4(B)). The amount of citrate excreted by roots of tolerant plants is almost two- to three-fold that observed for sensitive plants at low Al activity. At Al activities above 11×10^{-6} the roots of sensitive plants were severely damaged and organic acids may be liberated from the cytoplasm. This is consistent with results observed by Pellet *et al.* [9], indicating that citrate and malate are differentially excreted by Al-tolerant and Al-sensitive maize plants in response to Al treatment. Exudation of oxalate, succinate and isocitrate from roots of the Al-tolerant and Al-sensitive plants was not detected.

Since organic acid exudation could be due to Pi deficiency in the nutrient solution [11, 12], citrate exudation from roots of tolerant and sensitive plants in the presence and absence of 10 μM KH_2PO_4 at pH 4.1 was measured. The Pi-free nutrient solution did not stimulate citrate or malate exudation from the Al-tolerant plants selected from the Taiúba variety (results not shown). This result is in agreement with those observed by Delhaize *et al.* [6] and suggests a specific Al-inducing process involved in the Al tolerance in maize.

Al binds citrate more strongly [log $K_{\text{Al-citrate}} = 9.9$ [18] than malate [log $K^{+}_{\text{Al-malate}} = 5.34$ [19]. Thus, citrate is a better Al detoxifier than malate for the Al-tolerant plants of the maize variety Taiúba, but is the amount of citrate excreted from the roots sufficient to chelate all the Al ions in the test solution? By using the GEOCHEM-PC program [18] we estimated the free Al activity after citrate exudation from the tolerant roots in test solutions containing 10.3 and 50 μM $AlCl_3$. The free Al activity was reduced by 12 and 5% for the low and high Al concentration, respectively. The

reduction in the free Al activity for the low concentration is significant but cannot explain the exclusion process that alleviates the Al toxicity in the tolerant plants as the solution still contains free Al^{3+} in an activity sufficient to inhibit root growth. Perhaps the citrate concentration in the rhizosphere is maintained higher by association with the mucilage in the root apex [20]. This should prevent citrate diffusion from the rhizosphere, creating a barrier which prevents Al infiltration and thus increases the tolerance to the cation.

EXPERIMENTAL

Plant material. Al-tolerant and -sensitive plants were selected by two cycles of divergent selection in the maize variety IAC-TAIUBA based on root development of seedlings growing in nutrient solution containing 4.5 mg Al $litre^{-1}$ [21].

Growth solutions. Two solns were used: a complete nutrient soln was utilized to analyse the effect the Al and other trivalent cations in the primary root development [5] and a simple salt solution was utilized to analyse the organic acid exudation [9]. The complete nutrient soln contained (in μM): $Ca(NO_3)_2$, 500; KNO_3, 500; KH_2PO_4, 2; NH_4NO_3, 250; $MgSO_4$, 125; $Fe(NO_3)_3$, 2; $MnCl_2$ 2; H_3BO_3, 11; $ZnSO_4$, 0.35; $CuSO_4$, 0.2; Na_2MoO_4, 0.2. Al in the form of $AlK(SO_4)_2$ was added to the nutrient soln in concns ranging from 0 to 50 μM. In this solution the Al toxicity symptoms were observed for the sensitive and tolerant cultivars after 60 hr exposure to 24 and 48 μM Al, respectively. The simple salt solution contained 230 μM $CaCl_2$ and 230 μM NH_4Cl. Al in the form of $AlCl_3$ was added to the soln in concns ranging from 0 to 50 μM. The pH of both solns, with and without Al were adjusted to 4.1. Al associates with several inorganic ions to form complexes. Thus, it is important to estimate the free concentration of Al along with other ions which interfere with the Al toxicity in plants. The free activities of Al^{3+}, Ca^{2+} and Mg^{2+} were estimated by the GEOCHEM-PC program [18] (Tables 1 and 2). The activities of Ca^{2+} and Mg^{2+} were maintained constant to minimize their influence in the amelioration of Al toxicity [22].

Effect of Al, Ga *and* La *on the root development of maize seedlings.* Seeds were germinated at 30° between layers of filter paper saturated with complete nutrient solution at pH 4.1. When the roots reached *ca* 3 cm the seedlings were transferred to polystyrene holders which were then floated on 2 litres of aerated complete nutrient solution with and without 36 μM $KAl(SO_4)_2$, 150 μM $GaCl_3$ and 20 μM $LaCl_3$. The plants were incubated in a growth chamber with a photoperiod of 16/8 light/darkness at $26 \pm 1°$. The primary root length was monitored for 120 hr. The relative root growth (RRG) value was estimated by dividing the net root growth (final root length − initial root length) of the seedlings growing in the presence of Al, Ga or La by the net root growth of seedlings growing in the absence of the trivalent cations.

Table 1. Total concentration (c) and free activities (a) of Al^{3+} of and Ca^{2+} in the simple salt solutions containing $CaCl_2$ and NH_4Cl used to measure organic acid exudation in the presence and absence of $AlCl_3$ at pH 4.1. The GEOCHEM-PC program [18] was used to calculate the free cationic activities. For calculation of activity (a non-dimensional quantity), 1 mol dm^{-3} (1 M) was used as a standard. The concentration of the anion Cl^{-1} used to adjust the pH was included in the estimation of the activity of each cation

c/μmol dm^{-3}		$a \times 10^6$	
Al^{3+}	Ca^{2+}	Al^{3+}	Ca^{2+}
0	230	0	199.3
10.3	230	6.6	198.5
17.5	230	11.2	198.0
26.5	230	16.9	197.3
50.0	230	31.2	195.7

Determination of organic acid exudation from the root system. To prevent microbial degradation of compounds exuded by the roots, (especially short chain organic acid anions can be used as substrates for bacteria [6, 8, 23, 24]) all the procedures were conducted under sterile conditions. The seeds were surface sterilized for 1 min with ethanol and then for 30 min with 1% sodium hypochlorite (NaOCl). The excess NaOCl was washed out by rinsing several times with sterile distilled water. The seeds were germinated between layers of sterile filter paper saturated with simple salt solution for 48 hr at 30° and then transferred (10 or 20 seedlings per treatment) to 25 or 50 ml plastic flasks containing the simple salt solution, previously saturated with O_2 A series of $AlCl_3$ concns was added to achieve the free Al levels shown in Table 1. Seedlings were grown under aseptic conditions for 30 hr

Table 2. Total concentration (c) and free activities (a) of the cations in the complete nutrient solutions used to measure the effect of trivalent cations on the seminal root growth in the presence and absence of $AlCl_3$ at pH 4.1. The GEOCHEM-PC program [18] was used to calculate the free cationic activities. For calculation of activity (a non-dimensional quantity), 1 mol dm^{-3} (1 M) was used as a standard. The concentration of the anion Cl^{-1} used to adjust the pH was included in the estimation of the activity of each cation

c/μmol dm^{-3}			$a \times 10^6$		
Al^{3+}	Ca^{2+}	Mg^{2+}	Al^{3+}	Ca^{2+}	Mg^{2+}
0	500	125	0	391.0	98.5
12	500	125	5.3	389.5	97.7
24	500	125	10.1	385.7	96.8
36	500	125	15.2	384.6	96.6
48	500	125	19.6	382.5	96.1

without stirring and the pH of the solutions was kept at 4.0–4.2 by periodic addition of HCl under sterile condition. After the completion of the experiment the solutions were reduced by evaporation to 5–10 ml. Malate, citrate, oxalate, succinate and isocitrate were determined enzymically using small modifications of the procedures described by Delhaize *et al.* [6]. Reduction of NAD or oxidation of NADH was monitored by UV spectroscopy at 340 nm using a 5 cm optical path cuvet to increase the detection limit of the method. For malate, 2.0 ml of sample was incubated at 30° with 1.0 ml of buffer (0.5 M glycine, 0.4 M hydrazine, pH 9.0) and 50 μl of 30 mM NAD. After 30 min 5 μl of malic dehydrogenase (10 mg ml^{-1}, Sigma) were added. The reduction of NAD is directly proportional to the concentration of malate in the sample. For citrate, 2.5 ml of sample were incubated at 30° with 250 μl of Tris–Cl 1 M, pH 7.8, 5 μl of 28 mM NADH, 5 μl malic dehydrogenase and 5 μl lactic dehydrogenase (11 mg ml^{-1}, Sigma). After pre-incubation for 60–80 min, 5 μl of citrate lyase (140 mg ml^{-1} Sigma) were added. The oxidation of NAD is directly proportional to the citrate concentration in the sample. Oxalate, succinate and isocitrate were determined using kits from Boehringer–Mannhein and Sigma.

Effect of low phosphate on the exudation of organic acids. To verify if the level of phosphate influences the exudation of organic acids from maize roots, plants were grown in the simple salt solution with or without 10 μM KH_2PO_4. After 30, 40 and 50 hr, organic acids were measured as described earlier.

Hematoxylin staining of root apices. Roots of tolerant and sensitive plants that were grown in the complete nutrient solution in the presence and absence of Al were stained with hematoxylin according to procedure described by Delhaize *et al.* [5].

Acknowledgements—We would like to thank Dr Pedro Furlani of the Instituto Agronômico de Campinas, Brazil, for kindly providing the maize seeds. This work was financed by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and by Fundo de Apoio à Pesquisa-UNICAMP. PA received research fellowships from CNPq.

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