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Thin Layer Chromatography Analysis of Organic Acids in Maize (*Zea mays* L.) Plants under Al and Zn Toxicity

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Abstract: Thin layer chromatography analysis of organic acids from maize plants supplied with Al or Zn (500 and 1000 μM) is reported here to elucidate the role of organic acids in heavy metal sequestration. Organic acids detection reveals three spots in root extracts, corresponding to malonic (R_f 0.25), fumaric (R_f 0.33) and salicylic acid (R_f 0.78). Moreover, this analysis indicate the spots in shoot extracts corresponding to tartaric (R_f 0.17), malonic (R_f 0.26), malic (R_f 0.33), succinic (R_f 0.44) and salicylic acid (R_f 0.75). Al or Zn treatments generated very intense spot (R_f 0.75) that increased in intensity in root and shoot extracts, as the concentration of Al or Zn increased. TLC analysis revealed differences in spot intensities between the Al and Zn-treated plants. In roots, malonic acid accumulates more with Zn than with Al, in contrast to the shoots. In shoots Zn treatment at 500 μM caused a more increase in intensity of spot corresponding to malate, whereas, salicylic acid accumulation was much higher in the Zn-treated extracts at 1000 μM. Together, we hypothesize that organic acids may be involved in Al or Zn sequestration. The observations that Al generally produced more increase than Zn in the spot intensity in root and shoot extracts, suggest that organic acids are more involved in Al than in Zn detoxification.

Key words: Aluminum, fumaric acid, maize (*Zea mays* L.), malic acid, malonic acid, organic acids, salicylic acid, tartaric acid, thin layer chromatography, zinc

Introduction

Heavy metal pollution due to mining, smelting, manufacturing, agricultural or waste disposal technologies poses a serious environmental problem. Zn, an essential plant micronutrient, acts as an effective electron acceptor and donor in the active sites of many proteins involved in redox reactions and as cofactor of enzymes. Despite its importance to plant metabolism, Zn is toxic at high concentrations and Zn phytotoxicity has been shown to induce symptoms of oxidative injury (Weckx and Clijsters, 1997). On the other hand, Al is a ubiquitous element of the earth crust, representing approximately 7% of its mass. Although most of Al is incorporated into alminosilicates, low amounts of Al were present in solubilized forms that can affect the plant growth (May and Nordström, 1991). Acid soils cover some 40% of the earth's arable land and represent a major limitation to plant production (Foy, 1988; Taylor, 1988a, b). Al toxicity concerns only some

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of its soluble forms, where the most toxic monomers Al^{3+} prevail in acidic conditions (Kinraide and Parker, 1989; Delhaize and Ryan, 1995). The Al toxicity is manifest even at micromolar concentrations (Delhaize and Ryan, 1995), by an inhibition of the root growth (Sasaki *et al*., 1996) and results in poor uptake of water and nutrients (Kochian, 1995). Al toxicity led to characteristic toxicity symptoms, particularly at the root apical zone which gives rise to nodules-like structures (Foy *et al*., 1978). The mechanisms of heavy metal tolerance in plants have been reported for many plant species and evolve various metal homeostasis factors to control the cellular accumulation, distribution and sequestration of the heavy metals (Vulpe and Packman, 1995). Based on the site of metal detoxification and immobilization, two main mechanisms of Al tolerance have been proposed by Taylor (1991): external or exclusion mechanism, are those when the metal is prevented from entering the symplasm and reaching the sensitive intracellular sites and the internal tolerance mechanisms, when Al toxicity is limited by formation of stables complexes with organic ligands such as organic acids in the cytosol. These mechanisms could be also implicated in tolerance to other heavy metals such as Cd (Cobbett, 2000; Clemens *et al*., 2001; Vatamaniuk *et al*., 2001), Cu (Strange and Macnair, 1991), Zn (Schat *et al*., 2002; Hussain *et al*., 2004). The internal sequestration of excess metals is a common mechanism for metal accumulation which may play a role in the tolerance. The roles of several ligands have been reviewed by Rauser (1999). A number of metal-binding ligands that are recognized in plants to be involved in the chelation of toxic metals are mostly peptides or organic acids (Zenk, 1996; Larsen *et al*., 1998). The role of organic acids in Al detoxification has been reported in several studies (Taylor, 1988a, b; Delhaize *et al*., 1993; Pellet *et al*., 1995; Ryan *et al*., 1995). The organic acids are compounds of low molecular weight and have been shown to form stable and non toxic complexes with Al, either internally or externally (Delhaize *et al*., 1993; Pellet *et al*., 1995). Exudation of organic acids may protect root tips from Al induced injury, presumably by strongly binding surrounding toxic Al^{3+} ions. The exudation of organic acids, such as citrate and malate from root apices could be an efficient mechanism for Al exclusion and tolerance (Jones and Kochian, 1996) and is considered as a common feature of Al-excluding in resistant varieties of crop plants (Kochian, 1995; Barceló and Poschenrieder, 2002). Understanding these mechanisms will be an important aspect of developing plants as agents for the phytoremediation of contaminated sites (Salt *et al*., 1998). This study reports first result on thin layer chromatography separation of organic acids in *Zea mays* L. grown under Al and Zn stress.

Materials and Methods

Germination and Growth Conditions

Maize (*Zea mays* L.; var. LG 23/01) seeds were surface sterilized with 10% (v/v) H_2O_2 for 20 min, rinsed many times with tap water and germinated on filter paper moistened with distilled water in the dark, at 25°C for 3 days. The germinated seedlings were transferred to 12l basal nutrient solutions for 4 days. Selected plants of uniform size were selected and then transferred to identical solutions in 6l plastic pots (12 plants each) for 10 days. For treatment purposes, fourteen-day-old seedlings were transferred to solutions that contain 0 (control), 500 and 1000 μM Al or Zn, supplied as $Al(NO_3)_2.9H_2O$ and $ZnSO_4$, respectively for 4 days. The maize plants were grown in hydroponics as previously described by Chaffai *et al*. (2005). At harvest, the roots were rinsed three times with distilled water; the f. wt was determined and frozen in liquid N_2.

Organic Acid Extraction

The root and shoot samples were ground finely in ethanol using chilled mortar and pestle. The crude extracts were kept 24 h at 4°C (in the dark) and then filtered by passing through a glass fiber

filter and the residues were washed two times with ethanol. The ethanolic extracts which contain the organic acids were evaporated under low pressure, using rotate evaporator (Büchi). For Thin Layer Chromatography (TLC) analysis, the residues were dissolved in a known volume of EtOH:H_2O (20:2, v/v) mixture.

Thin Layer Chromatography (TLC) Analysis of Organic Acids

Basically, thin layer chromatography (TLC) consists of immobilized solid stationary phase coated thinly onto a glass plate and liquid mobile phase, which flows over the stationary phase. The organic acids are separated by adsorption process, on silica of the order of 0.25 mm thick on glass plate of 20×20 cm (Sigma). The plates are dried in an oven before use at 120C for 30 min. This serves to activate the adsorbent. The sample was applied as a narrow band to the stationary phase, 2 cm from the edge by means of a microsyringe. The solvent was removed from the band by gentle heating using of an air blower. Separation takes place in a glass tank that contains the developing solvent (mobile phase) to a depth of about 1.5 cm. This is allowed to stand for at least 1 h with a lid over the top of the tank to ensure that the atmosphere within the tank becomes saturated with solvent vapor. After equilibration, the thin layer is placed vertically in the tank so that it stands in the solvent. The system is kept at constant temperature whilst the development is occurring. Mobile phase, referred to as the eluent, consists of EtOH:NH_4OHcc:H_2O mixture (75.5:12.5:12, v/v/v) (Braun and Geenen, 1962), is then allowed to flow continuously over the stationary phase, resulting in the progressive separation of the organic acids. The plate developed and then removed from the tank and allowed to dry. A specific organic acid detection is to spray the plate with 0.4% (w/v) bromocresol green in ethanol, in which were added few drops of 0.1 N NaOH, which result in yellow spots (Copius-peereboom, 1969). The movement of compounds on TLC was characterized by specific R_f values, or retardation factor expressed as $R_f = d_A/d_F$ where d_A is the distance moved by the analyte (organic acid) from the origin and d_F is the distance moved by the solvent front from the origin. Organic acid identification is made on the basis of comparison of the movement of the organic acids with those of reference compounds chromatographed alongside the sample on the TLC plate. The amount of compound present in a given spot was quantified on the basis of spot intensity by means of densitometry.

Statistical Analysis

Data were statistically analyzed using one-way ANOVA and differences were considered significant at p0.05.

Results

Plant Growth under Al and Zn Stress

Based on morphological aspects, the plants exposed to Al stress exhibited noticeable root damages (Fig. 1). The plants grown in the presence of Al (500 and 1000 μM) manifest marked inhibition of the root elongation (Fig. 1) associated to severe reduction in the development of lateral roots which become very stunted, thicker and exhibited nodule-like structures (Fig. 1). Comparison of these symptoms and those observed with Zn stress at 1000 μM is showed in Fig. 2. The root elongation was more affected by Al than by Zn (Fig. 2). The Al and Zn at 1000 μM decreased by 40 and 30%, respectively, the root fresh weight (Table 1). However, the shoot fresh weight was similarly affected with both metals at 500 μM, whereas at 1000 μM, Zn treatment has more effect (Table 1). The results also indicated that Al increased markedly the shoot/root fresh weight ratio, whereas this ratio has tendency to decrease with Zn (Table 1).

Table 1: Effects of Al and Zn (500 and 1000 μM) on fresh weight of roots and shoots and the Shoot/Root fresh weight ratio in maize (...L.) seedlings. Values are means of 5 replicates±SE (n= 5)

Treatments		Roots (g FW)	Shoots (g FW)	Shoot/Root FW ratio
Control		1.00±0.11	3.26±0.28	3.36±0.14
Al (μM)	500	0.28±0.04*	1.32±0.12*	5.46±0.95
	1000	0.12±0.01*	1.09±0.05*	10.10±0.86
Zn (μM)	500	0.58±0.06*	1.29±0.09*	2.34±0.14
	1000	0.33±0.05*	0.75±0.08*	2.47±0.14

* Significant difference compared to the control (p 0.05) as determined by one-way ANOVA

Table 2: Retardation factor of reference organic acids (R) separated by thin layer chromatography (TLC)

Organic acids of reference	R
Oxalic acid	0.00
Citric acid	0.06
Tartaric acid	0.13
Malonic acid	0.27
Malic acid	0.30
Fumaric acid	0.36
Succinic acid	0.44
Salicylic acid	0.79
Benzoic acid	0.81

Table 3: Thin layer chromatography of organic acids from root extracts of maize (.....) seedlings in the presence of Al or Zn (500 and 1000 μM)

			Intensity of the spot				
			Control	Al		Zn	
Spots	Organic acids	R		500	1000	500	1000
S	Malonic acid	0.25	ND	+	++	++	+++
S	Fumaric acid	0.33	+	+++	++++	ND	ND
S	Salicylic acid	0.78	++	++++	+++++	+++	++++

+, ++, +++, ++++, +++++: indicated the yellow colour scale of the spot intensity compared to the control samples. ND: Not Detected

Table 4: Thin layer chromatography of organic acids from shoot extracts of maize (...L.) seedlings in the presence of Al or Zn (500 and 1000 μM)

			Intensity of the spot				
			Control	Al (μM)		Zn (μM)	
Spots	Organic acids	R		500	1000	500	1000
S	Tartaric acid	0.17	+	++	+++	+	++
S	Malonic acid	0.26	+	++++	+++	ND	++
S	Malic acid	0.33	+	++	++++	+++	+++
S	Succinic acid	0.44	+	+++	++++	++	+++
S	Salicylic acid	0.75	+	+++	++	++	+++

+, ++, +++, ++++, +++++: indicated the yellow colour scale of the spot intensity compared to the control samples. ND: Not Detected

Comparative TLC Analysis of Organic Acids in Roots Under Al and Zn Stress

The thin layer chromatography (TLC) separation of organic acids revealed 3 spots in root extracts, which were identified by comparison of their R_f to those of reference organic acids (R_{fR}) as malonate (S_1, R_f 0.25), fumarate (S_2, R_f 0.37) and salicylate (S_3, R_f 0.78) (Table 2 and 3). As shown in Fig. 3 and Table 3, the Al treatment induced qualitative and quantitative changes in the intensity of spots, with all spots exhibiting a strong intensity. The spot S_1 (malonate) which was not detected in control extract becomes apparent at 500 and 1000 μM Al (Table 3). The spot S_2 (fumarate) which

Fig. 1: Symptoms of Al toxicity in maize (*Zea mays* L.) roots. The maize seedlings were grown in hydroponics for 14 d and then treated with 500 and 1000 μM Al for 4 d

Fig. 2: Comparative effects of Al and Zn (1000 μM) on root elongation and morphology of maize (*Zea mays* L.) seedlings

showed faint intensity in the control plants exhibited higher intensity in roots of Al-treated plants (Table 3). However, the S_3 (salicylate) showed a much higher intensity compared to S_1 and S_2 spots in all extracts (Fig. 3 and Table 3). The main qualitative difference between the Al and Zn treatments was that S_3 increased in intensity much more in root extracts of Al-treated plants. Moreover, Zn has more important action than Al for the spot S_2 (malonic acid) (Table 3).

Comparative TLC Analysis of Organic Acids in Shoots under Al and Zn Stress

The shoot extracts recognize five spots which appeared as yellow spots (Fig. 4) and were identified on the basis of their R_f as tartarate (S_1, R_f 0.17), malonate (S_2, R_f 0.26), malate (S_3, R_f 0.33),

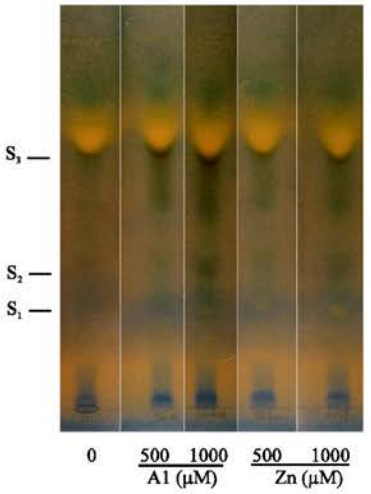

Fig. 3: Separation of organic acids from root extracts of maize (*Zea mays* L.) seedlings by thin layer chromatography (TLC) in the absence (control extracts) and the presence of Al or Zn (500 and 1000 μM). The organic acids were localized as yellow areas and identified by comparison of their R_f to those of reference organic acids that are separated alongside the sample

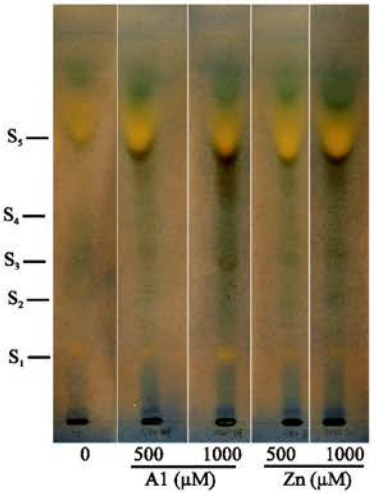

Fig. 4: Separation of organic acids from shoot extracts of maize (*Zea mays* L.) seedlings by thin layer chromatography (TLC) in the absence (control extracts) and the presence of Al or Zn (500 and 1000 μM). The organic acids were localized as yellow areas and identified by comparison of their R_f to those of reference organic acids that are separated alongside the sample

succinate (S_4, R_f 0.44) and salicylate (S_5, R_f 0.75) acids (Table 2 and 4). The S_2 was detected in extracts of control shoots, in contrast to what was observed in the root. The addition of Al caused an increase in the intensity of all spots, most notably in S_2 (malonate) at 500 μM and S_3 (malate)

and S_4 (succinate) at 1000 µM (Table 4). However, when Zn was added to the cultures, accumulation of organic acids, as indicated by yellow spots, follows the same pattern of increase in intensity as compared to Al, but to a lesser extent. By contrast, malate (S_3) and salicylate (S_5) spots exhibited a stronger intensity with Zn, at 500 and 1000 µM, respectively (Fig. 4 and Table 4).

Discussion

These results from the biomass determination provide strong evidence that Al is more toxic than Zn at both metal concentrations. The contrasting effects on the shoot/root fresh weight ratio suggest differences in the biomass allocation between roots and shoots under Al and Zn stress. Similar morphological symptoms have been previously reported under Al stress (Foy *et al*., 1978; Taylor, 1988a, b; Bennet and Breen, 1991). In maize plants, Al treatment inhibited the root growth and led to formation of callose at the subapical zone of the roots (Rasmussen, 1968). Since the root apices are well established as being a direct target for Al toxicity (Ryan *et al*., 1993), the perturbation of cell division at the meristematic apical zone (Horst *et al*., 1983) may result in inhibition of the root elongation observed in this study. Similar to our results, exposure to Al may have to be much longer for inhibition of the shoot growth to occur (Rengel, 1992). After Al exposure, the leaves of maize plants have been shown to exhibit red color which may result from phosphorus deficiency (Clark, 1977). Some factors such as plant specie, plant age or growth conditions may influence the occurrence of the Al toxicity symptoms (McQuattie and Schier, 1990).

The TLC analysis showed that the Al or Zn treatment led to elevation of the levels of organic acids detected in shoots or roots. This analysis indicated a primary role for organic acids in Al or Zn detoxification. Al-and Zn-mediated an increase in organic acid accumulation indicates that these compounds are probably involved in Al and Zn chelation or sequestration. One recurrent general mechanism for heavy metal detoxification in plants and other organisms is the chelation of the metal by a ligand and, in some cases, the subsequent compartmentalization of the ligand metal complex (Cobbett, 2000). The data described here provide evidence that accumulation of organic acids may prevent Al toxicity by complexation and are in agreement with organic acid analysis in maize roots, where higher amounts of aconitic and malic acids were detected in the roots of Al-treated plants than in control (Pellet *et al*., 1995). In wheat, fumaric, malic and succinic acid levels were found to increase in roots after Al treatement (Foy *et al*., 1990). The salicylic acid (SA) which was observed to increase in all extracts of Al and Zn-stressed plants, was found in mono- and dicotyledonous plants and has been shown to be involved in a number of natural processes such as flowering, senescence and stomata closure and nutrients absorption (Klessig and Malamy, 1994). Moreover, SA plays essential role in defense reactions in plants and is required for the systemic acquired resistance (SAR) (Gaffney *et al*., 1993). SA which synthesized in response to various environmental stimuli induced the accumulation of PR proteins (pathogenesis-related), peroxidases, SOD (superoxide dismutase) and membrane GRP (glycine-rich protein) (Enyedi, 1992). The GRP are structure proteins which intervene by reinforcing or repairing the cell walls (Bowles, 1990). Spraying of maize leaves with $Al(NO_3)_3$ has been shown to induce the accumulation of two types of GRP proteins (Bahloul, 1995). The SA may play a role in Al detoxification and to form complexes with Al (Dawson *et al*., 1986), but to a lesser extent than citrate or malate (Hue *et al*., 1986). The ability of organic acids to detoxify Al by formation of stable complexes has been studied by Hue *et al*. (1986). According to this author, the organic acids with short chains were grouped into 3 classes (I) the strong detoxifiers: citric acid, oxalic acid and tartaric acid; (II) the moderate detoxifiers: malic acid, malonic acid and salicylic acid and (III) the weak

detoxifiers: succinic acid, lactic acid, formic acid, acetic acid and phtalic acid. The capacity of organic acids to detoxify Al was related to the relative position of their OH/COOH groups in carbon chains. The strong detoxifiers had generally OH/COOH group's relied to adjacent carbons or possess two COOH groups directly connected (Hue *et al*., 1986). These positions allowed the incorporation of Al into cyclic structure by forming 5 or 6 stables bonds. The spot corresponding to salicylic acid S_3 can also represent benzoic acid (R_{fR} 0.81), but this organic acid has not been recognized in plants as playing a major role in heavy metal detoxification. However, it should be noted that salicylic acid and benzoic acid both derived from a common precursor: the cinnamic acid.

Furthermore, some but not all of organic acids detected in shoot extracts have been found in wheat (malate and succinate) (Foy *et al*., 1990) and bean (malate, malonate and succinate) (Lee and Foy, 1986). The Al has been shown to affect the organic acid concentrations in shoots of wheat (*Triticum aestivum*) (Foy *et al*., 1990). A decrease in the levels of aconitic acid and an increase of those of fumaric and malic acids were observed. Studying the quantitative changes in the content of organic acids in *Phaseolus vulgaris*, Lee and Foy (1986) observed an increase of the concentrations of succinic and malic acids, concomitantly to a decrease of that of citric acid in shoots of the tolerant cultivar. In the sensitive cultivar, they found an increase of the concentrations of succinic acid accompanied with a decrease of those of malic and citric acids. Together, present results indicate that accumulation of organic acids is a major component of heavy-metal detoxification processes but increased tolerance to metals may involve other mechanisms for heavy-metal detoxification. The organic acids are generally considered as agents of detoxification of heavy metals (Delhaize *et al*., 1993). Accumulation of organic acids was found in some Al resistant species (Barceló and Poschenrieder, 2002): oxalate and citrate in *Fagopyrum esculentum* (Polygonaceae) and citrate in *Cassia tora* (Caesalpinaceae). Their role is by metal sequestration and alleviation of toxicity (Taylor, 1991). The role of organic acids in the protection of the root apex from Al, in the detoxification of high tissue Al concentrations and their importance in the long-distance transport of Al from roots to shoots has been shown in *Fagopyrum esculentum* and *Hydrangea* (Ma *et al*., 1997a, 1998). Yet, it is well known that Zn could be stocked in vacuole as complexes with organic acids (Ernst, 1975). It has been suggested that the mechanism of Zn-tolerance was based on the chelation of Zn by malate in the cytoplasm and storage of Zn as various types of complexes in vacuoles. Similarly, Mathys (1977) have reported that Zn tolerance was associated with malate accumulation. Following the Zn tolerance model proposed by Mathys (1977), Zn was chelated by malate in the cytoplasm, transported to the vacuoles and accumulated there. The accumulation of citrate and malate has been reported in Zn-tolerant grasses of *Deschampsia caespitosa* grown in the presence of Zn (Thurman and Rankin, 1982; Godbold *et al*., 1984). Zn hyperaccumulation depends on the production of organic acids (citrate, oxalate) which chelate Zn during long-distance transport and storage (Salt *et al*., 1999). In cultured cells of *Nicotiana plumbaginifolia*, the Zn resistant cells accumulated higher levels of citrate and malate than the wild type cells (Kishinami and Widholm, 1987). The tolerance to Zn of *Nicotiana plumbaginifolia* cells was in part due to the ability to accumulate higher than normal levels of citrate and malate to chelate the toxic metal ions.

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