Citrate Exudation by Maize Roots

A possible mechanism of resistance to aluminium

Promotor: prof. dr. W. H. van Riemsdijk
hoogleraar in de bodemscheikunde en chemische bodemkwaliteit
Wageningen Universiteit

Co-promotor: dr. ir. W. G. Keltjens
universitair hoofddocent bij het Departement Omgevingswetenschappen
Wageningen Universiteit

Samenstelling promotiecommissie:

dr. J. A. C. Verkleij – Vrije Universiteit Amsterdam
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dr. ir. J. C. L. Meeussen – Energieonderzoek Centrum Nederland, Petten
prof. dr. P. C. Struik – Wageningen Universiteit

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Eduardo D. Mariano

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Abstract

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Low-molecular-weight organic anions have been intensively studied as possibly involved in a mechanism of Al resistance in plants, due to their involvement in many metabolic processes and to their negative charge, conferring them the capacity to complex metals. The objective of the thesis was to study the root exudation of organic anions as a potential mechanism of Al resistance operating in maize (*Zea mays* L.). The effect of Al exposure on root organic anion exudation was studied with roots grown under sterile conditions, with maize genotypes that differ in sensitivity to Al. Citrate accounted for the majority of the organic anions exuded, followed by malate, *trans*-aconitate, fumarate, and *cis*-aconitate. Along the longitudinal axis of fully developed seminal roots citrate was exuded mainly in the regions of root apices, either belonging to the main root or to the lateral roots in the most basal part of the main root. Rates of citrate exudation from root apices of eight genotypes exposed to Al correlated significantly well with their relative Al resistance; a less inhibited root elongation accompanied higher exudation rates.

The effects of Al on nutrient uptake were also studied in the thesis. The spatial localisation of nutrient uptake on the root axis of maize seedlings was assessed and revealed that Al is affecting nutrient uptake widely along the longitudinal axis of the root. Compared with the pattern of citrate exudation along similar axes it seems that citrate is probably primarily involved in making plants resistant to Al by detoxifying Al around the root meristems, the most sensitive part for root growth. Local citrate exudation does not seem to be directly involved in nutrient uptake, because the segment with the highest citrate exudation (the apex) shows almost no nutrient uptake, while the root zone with the highest nutrient uptake shows almost zero citrate exudation.

The question whether quantitatively the amounts of these small ligands released in the root environment are adequate to explain resistance to Al was tackled by combining experimental and modelling work. The results of the simulations strongly support the notion that citrate indeed can underlie Al resistance in maize. For the conditions considered in this study, detoxification of apoplastic Al and protection of this compartment seem more realistic and more important than those in the interface root-outer solution.

Contents

Chapter 1

General introduction

A complex of factors can restrict plant growth in acid mineral soils. In general, in moderate acid soils the plant supply with nutrients such as phosphorus (P), potassium (K), magnesium (Mg), and calcium (Ca) is often marginal or even deficient, whereas at high soil acidity aluminium (Al) toxicity is mostly the dominant growth limiting factor (Keltjens, 1997; Tan et al., 1991). During acidification of mineral soils phytotoxic forms of Al are released into the soil solution to concentrations that generally affect plant functioning and plant growth (Foy, 1984). The harmful effects of excessive toxic Al occur primarily in the roots. These effects are a rapid inhibition of root growth and disruption of nutrient uptake. Therefore Al toxicity is often expressed simultaneously in two ways, namely reduced root growth and induced deficiency of mineral nutrients (e.g. Ca, Mg, and P; Tan et al., 1992). Inhibition of root growth particularly leads to shallow root systems, low root densities, and poor exploitation of the soil. Consequently, plants become more susceptible to other abiotic stresses such as water and nutrient shortage (Foy, 1984).

Amelioration of soil acidity stress can be reached by liming the soil and/or improving its fertiliser supply (Keltjens, 1997; Miranda and Rowell, 1987; Rao et al., 1995). An alternative or supplemental approach to reduce the detrimental effects of soil acidity on plant growth is the use of plant species or cultivars that show superior resistance to Al. Considerable progress has been made in identifying Al resistant germplasm and efforts to select and breed such plant material are being made worldwide with several crop species (e.g. maize, Granados et al., 1993; Magnavaca et al., 1987; sorghum, Furlani and Clark, 1981; Gourley, 1987; wheat, Scott and Fisher, 1989). An important part of the proposed plant breeding approach is the determination of plant genetic, physiological, and biochemical mechanisms by which plants resist to mineral stress, including excess soluble Al in acid soils.

Mechanisms of aluminium toxicity and aluminium resistance

A significant part of the current research on plant metal toxicity has focused on the mechanisms of Al phytotoxicity and Al resistance, indicating the relevance and agronomic importance of this metal toxicity problem (Kochian, 1995). Aluminium hydrolyses in solution such that the trivalent Al species, Al^{3+}, dominates in acid conditions (pH < 5), whereas the $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ species are formed with increasing soil pH. Many of these monomeric Al cations bind to various inorganic and organic ligands such

as PO_4^{3-}, SO_4^{2-}, F^-, organic anions, proteins, and lipids (Kinraide, 1991). With this wide range of interactions, Al has been shown to disturb several biochemical and physiological processes and consequently many mechanisms of Al toxicity have been proposed. These mechanisms include Al interactions within the root cell wall, Al disruption of the plasma membrane (PM) and its transport processes, and Al interactions with symplastic constituents such as calmodulin (for a review see Delhaize and Ryan, 1995; Kochian, 1995; Rengel, 1992a). However, there is no consensus on the cellular site of Al toxicity. Because Al can bind and precipitate readily within the root cell walls and because it has an apparent slow rate of penetration into the symplast, it has been hypothesised that the toxic effects of Al might be expressed through extracytosolic lesions such as disruption of normal functioning of the plasma membrane (Rengel, 1992a), or that the toxicity is mediated through ion transporters or signal-transduction events initiated at the outer surface of the plasma membrane (Kochian, 1995; Rengel, 1992b, 1996). The primary Al effects are very fast and may therefore occur while Al is still in the Donnan free space and on the apoplastic side of the plasma membrane. Long-term responses are not necessarily caused by Al directly but may rather be a consequence of Al-related impairment of numerous other biochemical and physiological processes (Rengel, 1992a).

Reduction of root growth observed in the presence of Al results probably from both inhibition of root cell elongation and inhibition of root cell division (Marschner, 1991). During the initial stages of Al inhibition of root growth, Al interactions with cell elongation must play a primary role whereas Al inhibition of cell division and DNA synthesis are expected to play a role in sustained root growth inhibition after the first 24 h of Al exposure (Kochian, 1995). The Al-induced inhibition of root growth forms the basis of many of the rapid tests for Al resistance in plants.

Aluminium also interferes with the uptake, transport, and use of water and several mineral nutrients by plants, by either damaging the root or by exerting antagonistic effects on nutrient absorption. In the root apoplast, a major site of Al accumulation (Godbold and Jentschke, 1998), Al inhibits nutrient uptake by blocking ion channels on the plasma membrane, strongly competing with divalent cations (e.g. Ca, Mg) for binding sites at the root cortical cell walls, and precipitating P (Clarkson, 1967; Keltjens, 1995; Rengel and Robinson, 1989a,b; Tan and Keltjens, 1990).

Several mechanisms have been proposed in the literature to explain the plant Al resistance. These mechanisms can be divided into two types depending on whether the site of Al detoxification is inside or outside the symplast (Kochian, 1995; Taylor, 1991). Mechanisms facilitating Al exclusion from the symplast of root cells (Al exclusion mechanisms) are those mechanisms where metals are prevented from crossing the plasma membrane, entering the symplast, and reaching sensitive intracellular sites. Mechanisms conferring the ability of plants to tolerate Al in the plant symplast are called

Al tolerance mechanisms (Kochian, 1995). Exclusion mechanisms might include Al immobilisation at the cell wall, selective Al permeability of the plasma membrane, a plant-induced pH barrier in the rhizosphere or apoplast, exudation of chelate ligands, exudation of phosphate, and Al efflux (Foy, 1988; Taylor, 1988, 1991). It is not clear how these mechanisms are related and which may be the most important in attempting to explain Al resistance.

Al is clearly immobilised at the root-soil interface, but the extent to which exclusion plays a role in the physiology of Al resistance is not known (Taylor, 1988). Exudation of organic compounds that can chelate Al in the root rhizosphere has been described as a possible mechanism underlying Al resistance of certain plant species or cultivars. Certain plants seem to have the potential to release great amounts of low-molecular-weight organic anions into the rhizosphere that can chelate and detoxify Al and such an exudation of organic anions might substantially contribute to the Al resistance of certain species or cultivars (Delhaize et al., 1993; Jones, 1998; Keltjens, 1997). This mechanism seems more realistic once organic ligands, produced in and subsequently released by root cells, can be expected to accumulate even to higher concentrations in the root apoplast than in the rhizosphere (Keltjens, 1997). One of the main questions in this research is whether observed root flux densities of such organic compounds along the root can be high enough to chelate and thus detoxify a major part of the phytotoxic monomeric Al locally present in the root apoplast and/or rhizosphere.

Organic anions in the plant and in the soil

Plant roots contain many organic anions varying in chain length with lactate, acetate, oxalate, succinate, fumarate, malate, citrate, isocitrate, and aconitate being the main components (Jones, 1998; Marschner, 1995). Some of these (e.g. citrate, malate, and fumarate) are present in all living cells as intermediates of the tricarboxylic acid cycle (TCA cycle), which is also called the Krebs cycle. They are carbon compounds that possess at least one carboxyl group and at the near-neutral pH of the cytoplasm, most of these carbon compounds exist as fully dissociated anions. These compounds are almost certainly released by the roots as anions (dissociated from protons) and not as acids (Ryan et al., 2001). Their release from roots depends presumably on their concentration in the root tissue as well as on the permeability of the root cell membranes (Kraffczyk et al., 1984).

Organic anions are a common component of root exudates and thus are often found in soil solutions. Generally higher concentrations are found in rhizosphere soil than in bulk soil (Jones, 1998). After going from the root cell to the outside root solution, organic anions may interact with a wide range of soil constituents. Due to the negative charge associated with their carboxyl groups, they can readily and rapidly form com-

plexes with metal ions in solution or adsorb to the soil solid phase. Besides, they can be used by soil microorganisms as a source of energy or be leached out of the soil profile by percolation water.

Organic anions have the capacity to form complexes with metal ions in (soil) solution and thus reduce metal ion activity. The degree of complexation, however, depends on the particular organic acid involved, the concentration and type of metal and the pH of the (soil) solution (Mench and Martin, 1991). Organic anions like malate, citrate, and oxalate all have a high affinity for trivalent metals such as Al^{3+} and Fe^{3+}, with maximal complexation occurring at pH 4.0–4.5 (Motekaitis and Martell, 1984). The consumption of organic anions by microorganisms is probably an important process reducing their effectiveness in detoxifying metals (Ryan et al., 2001). However, the biodegradation of organic anions in the soil appears to be highly dependent on the amount and type of sorption to soil particles, with Al and Fe hydroxides providing the greatest protective effect (Jones et al., 1996b; Jones and Edwards, 1998).

Aim of the research and outline of this thesis

The general aim of the research reported in this thesis was to study the root exudation of organic anions as a potential mechanism of Al resistance operating in maize (*Zea mays* L.). As reported above, under conditions of Al toxicity some plant species seem to release organic anions from roots as a mean of protecting themselves against the toxic effects of excess Al. It is suggested that the exuded organic anions protect the plant by chelating and detoxifying Al in the root apoplast and/or rhizosphere (Miyasaka et al., 1991).

In Chapter 2 genotypic variation for Al resistance in maize was studied with a collection of ten maize genotypes obtained from the National Maize and Sorghum Research Centre (CNPMS), EMBRAPA, Brazil. Three screening techniques based on culture solution and soil as rooting medium were used to assess the relative Al resistance of these genotypes and to rank them according to this feature. Using the two genotypes representing the extremes of Al sensitivity within the collection that was available to us, the effect of Al exposure on root organic anion exudation was studied with roots grown under sterile conditions, both qualitatively and quantitatively in Chapter 3. The major low-molecular-weight organic anions exuded by roots when exposed to Al were identified and the rates of organic anion exudation as well as the spatial distribution of the exudation along the root axis were determined. A first qualitative evaluation of the possible role of citrate in protecting the roots from the effects of Al on root elongation and on nutrient uptake is given.

Chapter 4 focuses on the effects of Al on the root status of citrate, the principal organic anion exuded by maize roots in response to Al exposure. This chapter combines a literature review about the effects of Al on processes involved in the dynamics of citrate in plant roots (e.g. synthesis, reallocation, storage, exudation) with experimental work done with an Al resistant maize genotype to test some of the theories described in literature.

In Chapter 5 the effects of external Al on nutrient uptake were studied with the ten maize genotypes showing significant differential sensitivity to Al. Plants of the different maize genotypes were tested in culture solution containing Al to check whether they indeed differ in uptake of macro and micronutrients when exposed to Al and whether variation in nutrient uptake among genotypes, due to Al exposure, corresponds with variation in Al resistance as described in Chapter 2. Due to the relatively long period of exposure to Al (14 d), the reduction of nutrient uptake caused by Al observed in this study was probably a combined result of direct, or primary, effects of Al on nutrient uptake and of indirect, or secondary, effects on root and shoot growth, affecting plant's uptake capacity (root) and plant's nutrient requirement (shoot).

Because the primary effects of Al on nutrient uptake cannot be easily separated from its secondary effects, short-term (~24 h) experiments were established to study only the direct effects of Al on nutrient uptake by maize roots (Chapter 6). The effects of Al on the uptake of Ca, Mg, K, and P by whole roots as well as by distinct regions of the main seminal root were studied with two maize genotypes exhibiting significant differential resistance to Al. The investigation on the spatial distribution of the Al effects on nutrient uptake (Chapter 6) and on citrate exudation (Chapter 3), revealed zones of the root that are more sensitive to Al, allowing a qualitative evaluation of the potential role of organic anions in a mechanism of protection against the adverse effects of Al specifically on nutrient uptake.

A quantitative approach to evaluate the role of citrate released by roots in complexing Al is presented in Chapter 7. The citrate exudation rates measured experimentally in Chapter 3 were used in the calculations of citrate build-up in the root apoplast and outer root solution. Complexation of Al at local root conditions was calculated using a speciation model. The thesis concludes with an evaluation of the significance of the results presented and with a discussion of the prospects for future research (Chapter 8).

Chapter 2

Variation for aluminium resistance among maize genotypes evaluated with three screening methods[♣]

Abstract – Genetically determined differences in aluminium (Al) resistance exist among plant species and genotypes, and efforts to select and breed maize germplasm with higher resistance to Al have been made worldwide. This work aimed to study genotypic variation for Al resistance in maize genotypes using three different screening techniques, to compare the results of the screening techniques, and to select genotypes with differential sensitivity to Al for further research on the mechanisms of Al resistance in maize. The effects of Al on various plant characteristics were studied with ten maize genotypes in a series of experiments that comprised short-term (4 and 14 d) exposures to Al in culture solution (up to 100 μM Al) as well as longer-term (40 d) growth in an acid soil (soil solution pH range 3.4–4.1). Al resistance varied widely among the maize genotypes, as revealed by the different screening techniques used. A screening method based on root elongation rate of seedlings growing in culture solution was effective in discriminating resistance to Al. A concentration of 40 μM Al gave the best differential responses among the ten genotypes studied causing reductions in root elongation rate of 10% to 68%. The best indicators of differential Al resistance were root characteristics, especially root length. Internal root concentrations of citrate and malate, however, did not reflect plant resistance to Al. The Al resistance rankings established with the screening techniques were consistent and indicated genotypes with contrasting sensitivity to Al to be used in further studies of mechanisms of Al resistance in maize.

Key words: acid soil, aluminium toxicity, culture solution, organic anions, root elongation rate, *Zea mays* cultivars

[♣] with Willem G. Keltjens

Introduction

Soil acidity is one of the major problems for crop production in many parts of the world. Constraints for plant growth often associated with soil acidity are low nutrient availability and high concentrations of toxic aluminium (Al). Al particularly inhibits root elongation, leading to shallow root systems, low root densities, and poor exploitation of the soil. Consequently, plants become more sensitive to other abiotic stresses such as water and nutrient shortage (Foy, 1984). Maize (*Zea mays* L.) cultivation in acid soils is generally negatively affected, mainly due to toxicity of Al and manganese, and deficiency of calcium, magnesium, phosphorus and zinc. Increased ear rot percentage, poor plant vigour, delayed plant maturity, and reduced grain yield were associated with increased degrees of Al stress in maize (Kasim et al., 1990).

Addition of lime is commonly recommended to raise soil pH, lower soil exchangeable Al, and improve plant growth. However, due to the high quantities of lime often needed, this practice is not always economically feasible (Foy et al., 1987). Plant species, and cultivars within species, differ widely in resistance to Al, and selection and breeding of plant germplasm resistant to Al appear an alternative approach to overcome some problems related to toxic effects of Al in acid soils.

Considerable genetic variability in Al resistance has been reported in maize among inbred lines, hybrids and varieties. This genetic variability has been assessed using several characteristics like visual toxicity ratings, shoot and root dry matter yields, root staining, total root growth, and actual and relative root length (root length with Al stress divided by root length without Al stress). Screens of maize germplasm for resistance to Al were mainly made in field experiments, where plants were grown on acid soils (Granados et al., 1993; Pandey et al., 1994), and in greenhouse experiments, where plants were grown either in pots with acid soil or in culture solution with Al (Kasim and Wassom, 1990; Lopes et al., 1987; Magnavaca et al., 1987; Urrea-Gómez et al., 1996). On a short-term, Al appears to affect root growth more severely than shoot growth, and therefore root characteristics are often used as Al resistance indicator in screening studies (Furlani and Clark, 1981). In culture solution studies the best root characteristic to discriminate inbred lines and varieties of maize for resistance to Al was root length, as Al concentrations showed a linear effect on root length (Kasim and Wassom, 1990; Magnavaca et al., 1987). Under field conditions, however, grain yield, relative to the average of a group of selected varieties, was the best indicator of differential Al resistance (Kasim et al., 1990). Another possible indicator of Al resistance in plants might be the root concentration of organic anions. It is hypothesised that roots containing large amounts of organic anions are more likely to release these compounds into the rhizosphere where

they will form complexes with Al reducing its toxicity to root membranes and facilitating its absorption by roots and immobilisation in the plant (Jones, 1961).

The different approaches used to assess Al resistance have been responsible for many conflicting results in genotypic differences in resistance to Al. Germplasm grown under controlled laboratory conditions do not perform similarly when grown under less controlled field conditions (Duncan et al., 1983) and this lack of correlation may be caused by a significant interaction between genotypes and the stress conditions imposed.

The objectives of this study were (*i*) to study genotypic variation for Al resistance among maize genotypes using three different screening techniques, (*ii*) to compare the results of the three techniques, and (*iii*) to select genotypes with contrasting resistance to Al for further research on the mechanisms involved in resistance of plants to Al.

Materials and methods

Plant material

The genotypes used in the experiments reported here belong to the collection of the maize breeding program of the National Research Centre of Maize and Sorghum – EMBRAPA, Brazil. Ten Brazilian maize genotypes among varieties and hybrids were selected to represent a range of resistance to Al. The selection was based mainly on results from previous screening experiments carried out at EMBRAPA in culture solution containing different concentrations of Al. Their names are: G1 (64×1143; single cross hybrid); G2 (BR201-M; single cross hybrid); G3 (HD91102; double cross hybrid); G4 (13×1143; single cross hybrid); G5 (CMS36; open-pollinated variety); G6 (11×723; single cross hybrid); G7 (20×723; single cross hybrid); G8 (HD9148; double cross hybrid); G9 (BR106; open-pollinated variety); and G10 (20×22; single cross hybrid). Subsequently, they will be referred to as G1, G2, G3, etc.

These maize genotypes were tested on their sensitivity to Al under greenhouse conditions through a series of three experiments described below.

Root Elongation Assay

Above described ten maize genotypes were used in a short-term nutrient solution experiment to determine their relative resistance to Al on the basis of root elongation. Seeds were germinated on filter paper moistened with 1.0 m*M* $CaSO_4$ solution in the dark at 25 °C for 4 days. Seedlings were then transferred to 150-L containers, containing a basal nutrient solution with pH 4.0 and chemical composition (m*M*): 1.0 NH_4NO_3, 0.005 NaH_2PO_4, 0.5 K_2SO_4, 0.5 $CaCl_2$, 0.125 $MgSO_4$, and (μM): 46 B, 0.3 Cu, 286 Fe (as Fe-EDTA), 0.1 Mo, 9.2 Mn, 0.8 Zn. Seedlings were fixed in strips of foam floating

on the aerated nutrient solution. Only the main seminal root was allowed to develop whereas eventual seminal and adventitious roots were cut off immediately after appearance. After 3 days of growth in the basal nutrient solution, uniform seedlings of each genotype were selected and subsequently randomly transferred to 50-L containers holding five Al treatment solutions (0, 10, 20, 40, and 100 μmol $AlCl_3$ L^{-1}). Before mounting the seedlings in plastic discs suspended over the nutrient solution, their root length was measured with a ruler. The seedlings were kept in the Al treatment solutions for 96 hours and had their root length measured every 24 hours.

The pH of each container was checked daily and, when necessary, adjusted to the initial value of 4.0. The nutrient solution of each container was sampled daily and analysed on P, K, Ca, Mg, Cu, Fe, Mn, Zn and Al to monitor the composition. No adjustments were needed. Chemical analyses of the nutrient solution were carried out by ICP–AES.

The treatment design was a factorial combination of basal nutrient solutions containing five concentrations of Al and ten maize genotypes. An experimental split-plot design with three replicates was used, with the Al concentrations randomly assigned to whole plots and the maize genotypes randomly assigned to subplots. Each nutrient solution container (50 L) constituted a whole plot and included ten subplots of four plants each. The subplot mean of four plants constituted the experimental unit.

After finishing the experiment, data of root length measurements were submitted to linear regression in order to describe the root elongation under Al stress through the experimental time (0 to 96 hours). A first-order polynomial equation ($y = a + bx$) was fitted between root length and time of exposure to Al for each experimental unit. The slope of the equation (root elongation rate, mm h^{-1}) was further used in the analysis of variance and mean tests using the General Linear Models procedure of SAS (SAS Institute, 1990). The genotype × Al interaction sum of squares was partitioned into single degree of freedom orthogonal contrasts, where Al concentrations were studied in simple effect comparisons within each genotype. To compare concentrations of Al (main plot) within a given genotype (subplot) the Satterthwaite Approximate *F* Test was used (Neter et al., 1996).

Soil Test (Pot experiment)

This experiment was carried out to assess the Al resistance of the ten maize genotypes when grown in acid soil. The maize genotypes were grown in a greenhouse in pots containing an acid sandy soil from a forest near Ede, The Netherlands. The soil was collected, air-dried and sieved through a 5-mm mesh. Before fertiliser and lime were applied the soil had an average pH-$CaCl_2$ = 3.4, P-$CaCl_2$ = 0.2 mg kg^{-1} soil and organic matter = 103 g kg^{-1}. To create a range of soil pH and soluble soil Al, four rates of lime

(0.0, 0.5, 1.0, and 1.9 g $Ca(OH)_2$ kg^{-1} soil) were applied to portions of 2.5 kg of dry soil. Subsequently after basal fertilisation with (mg kg^{-1} soil) 300 N, 80 P, 200 K, 40 Mg, 0.1 B, 0.004 Cu, 1.04 Fe, 0.002 Mo, 0.1 Mn, and 0.01 Zn, the soil was moistened to field capacity and incubated in plastic bags for 40 days. The rates of lime application were chosen from a titration curve obtained by an incubation test with $Ca(OH)_2$.

After incubation the soil was transferred to 2.5-L plastic pots and covered with a layer of 3 cm of quartz sand. The maize seeds were sown and germinated in this sand layer to avoid influence of the soil acidity on the germination and initial establishment of the seedlings. One week later the number of seedlings was thinned to 3 per pot. Three pots per lime rate were left without plants and used as control (blanks). The soil moisture was maintained at field capacity with demineralised water throughout the experimental period by daily weighing and allowing for increasing plant fresh weight. The experiment was carried out in summer and plants were harvested 40 days after sowing. The roots were separated from the soil and washed. One part of the total fresh root material was taken to measure total root length on a Comair root length scanner (Hawker De Havilland, Melbourne, Australia). Root material was oven-dried at 70 °C for 72 hours, and weighed. The specific root length (SRL) was calculated by dividing the root length of a sample by its dry matter weight.

At harvest soil solutions were collected by centrifugation of soil samples. Soil solution pH was measured immediately and total concentrations of Al, P, and basic cations in soil solution were determined by ICP–AES. Dissolved organic carbon was measured in soil solution samples on a SK^{12} TOC/DOC analyser (Skalar, Breda, NL).

The experimental design was a completely randomised one with three replicates. The treatments resulted from a factorial combination of four rates of lime and ten maize genotypes. Analysis of variance was performed for all plant characteristics measured using the General Linear Models procedure of SAS (SAS Institute, 1990). When significant, the genotype × Al interaction was partitioned into single degree of freedom orthogonal contrasts, where Al concentrations were studied in simple effect comparisons within each genotype.

Culture Solution Test (Pot experiment)

Plants of the ten maize genotypes were also tested on their resistance to Al in a culture solution technique at zero and 100 μM Al. The experiment was conducted in pots filled with continuously aerated culture solution with pH 4.0 and chemical composition (m*M*): 2.0 NH_4NO_3, 0.0375 NaH_2PO_4, 1.0 K_2SO_4, 1.0 $CaCl_2$, 0.25 $MgSO_4$, and (μM): 46 B, 0.3 Cu, 286 Fe (as Fe-EDTA), 0.1 Mo, 9.2 Mn, 0.8 Zn.

Seeds were germinated in moist quartz sand for 7 days. After germination, the roots were washed to remove the sand and a total of 54 uniform seedlings per genotype was

used for the experiment. Seedlings were wrapped loosely with sponge rubber and mounted in holes of plastic covers (discs) placed on 6-L pots (9 seedlings pot^{-1}). After a pre-growth for 4 days in the above mentioned culture solution the Al treatments were imposed. The culture solution was replaced by a similar culture solution containing 100 μmol $AlCl_3$ L^{-1} or without Al, used as a control. The culture solutions were renewed every other day and 14 days after the Al treatment had started the plants were harvested. The 9 plants from each pot were divided into: 4 plants for root length measurement, 4 plants for shoot and root dry matter, and 1 plant for organic anion analysis in the root material. The shoots and roots were oven-dried at 70 °C for 72 hours, and weighed. Root length measurements were carried out on representative sub-samples of fresh root material using a Comair root length scanner (Hawker De Havilland, Melbourne, Australia). The specific root length (SRL) was calculated by dividing the root length of a sample by its dry matter weight.

For the analysis of organic anions in the root material, enzymatic methods were used (Boehringer Mannheim, 1984). Samples of 100 mg of dry fine-ground roots were extracted in 20 mL of demineralised water for 30 minutes in a shaker. The extracts were filtered and immediately analysed on malate and citrate.

The experimental design was a completely randomised one with three replicates. The treatments resulted from a factorial combination of two Al concentrations (0 and 100 μM Al) and ten maize genotypes. Analysis of variance was performed for all plant characteristics measured using the General Linear Models procedure of SAS (SAS Institute, 1990). When significant, the genotype × Al interaction was partitioned into single degree of freedom orthogonal contrasts, where Al concentrations were studied in simple effect comparisons within each genotype. To study the Al resistance as expressed by the ratio [+Al/−Al], the data were log-transformed, since [log(+Al/−Al)] = [log(+Al) − log(−Al)]. Using the ‘estimate’ statement of General Linear Models procedure of SAS (SAS Institute, 1990), these differences were calculated and the values were further compared using the Tukey test.

Results

Root Elongation Assay

Distinct root elongation rates (RER) were observed among the genotypes growing in nutrient solution with and without Al, which resulted in a highly significant genotype × Al interaction. Taking into account the natural genotypic variation in RER, comparisons were always done between Al concentrations within each genotype instead of comparing

RER values of different genotypes at each Al concentration. These comparisons revealed a wide range of Al resistance among the ten genotypes studied (Table 1).

Table 1. Root length at the beginning of Al treatment (intercept) (± standard error) and Root Elongation Rates (RER) of maize genotypes grown for 96 hours in culture solution containing various concentrations of Al. Within each genotype, only values marked with ** ($p < 0.01$) or * ($p < 0.05$) are significantly different from the control (Al 0)

Group/Genotype	Intercept	Root Elongation Rate				
		Al 0	Al 10	Al 20	Al 40	Al 100
I. Resistant	mm			mm h^{-1}		
G4	141.7 ± 4.4	1.277	1.482 (16)[1]	1.043 (18)	1.150 (10)	0.750 (41)**
G1	91.4 ± 2.5	1.368	1.311 (04)	1.153 (16)	1.195 (13)	0.728 (47)**
G5	93.4 ± 2.9	1.270	1.366 (08)	1.330 (05)	0.938 (26)*	0.772 (39)**
Group Mean			(07)	(13)	(16)	(42)
II. Intermediate						
G3	149.8 ± 3.5	1.416	1.443 (02)	1.218 (14)	0.841 (41)**	0.434 (69)**
G6	89.4 ± 2.3	1.451	1.524 (05)	1.290 (11)	0.765 (47)**	0.294 (80)**
G2	45.0 ± 1.9	0.845	0.848 (<1)	0.665 (21)	0.436 (48)**	0.153 (82)**
G10	136.7 ± 2.3	0.865	1.073 (24)	0.741 (14)	0.409 (53)**	0.209 (76)**
Group Mean			(08)	(15)	(47)	(77)
III. Sensitive						
G8	158.2 ± 3.1	1.589	1.424 (10)	1.207 (24)**	0.740 (53)**	0.340 (79)**
G9	113.3 ± 2.9	1.343	1.274 (05)	0.968 (28)**	0.538 (60)**	0.212 (84)**
G7	104.3 ± 1.4	1.214	1.276 (05)	0.780 (36)**	0.391 (68)**	0.185 (85)**
Group Mean			(03)	(29)	(60)	(83)
Overall Mean			(04)	(19)	(42)	(68)

[1] Values between parentheses are percentage reduction or increase (underlined values) in RER

With exception of the lowest concentration of Al (10 μM Al), the Al treatments caused a general reduction in RER, and consequently, in root length of the maize plants.

Averaged across the ten maize genotypes, the reduction in RER varied from 19%, at 20 μM Al, to 68%, at 100 μM Al. When growing at 10 μM Al in solution, most genotypes showed a beneficial effect of Al on root elongation, reaching up to 24% of increase in RER.

The ten genotypes were divided into three groups according to their similarity in sensitivity to increasing concentrations of Al (Table 1). The differences among groups could be illustrated very clearly. While group III showed a significant reduction in RER of 29% at 20 μM Al, group I only showed a significant reduction in RER (42%) when 100 μM Al was applied. This average reduction of 42% with plants of group I was comparable to the reduction of 47% caused by 40 μM Al with plants of group II. Although all genotypes showed a significant reduction of the RER when growing at 100 μM Al, G4 and G5 were less affected than the other genotypes.

At 40 μM Al the differences in Al sensitivity among genotypes were highest as the greatest variation in RER was observed. Showing a similar RER in the absence of Al, G4 had the lowest (10%) and G7 had the highest (68%) reduction in RER at 40 μM Al compared to their controls.

Al concentrations lower than 40 μM were too low to induce sufficient large changes in root elongation with all ten genotypes. At 10 μM Al in solution, RER of several genotypes was even higher than in the absence of Al. Based on RER values at 20, 40, and 100 μM Al in solution (Table 1), differences in Al sensitivity among groups were clearly shown. With significant root impairment at Al concentrations $\geq$ 20, $\geq$ 40, and $\geq$ 100 μM, groups III, II, and I represent maize genotypes sensitive, intermediate, and resistant to Al, respectively.

Soil Test

The chemical characteristics of the soil solution after application of lime and after cultivation are shown in Table 2. As expected, lime applied to the soil produced a range in soil solution pH and soluble Al. Soil solution pH varied from 3.4 (unlimed) to 4.1 (highest lime rate), with corresponding Al concentrations in soil solutions decreasing from 1278 to 111 μmol Al L^{-1}. Improved growth of plants with higher lime application caused higher nutrient uptake and consequently lower final concentrations of K, Ca, and Mg in soil solution, but had no significant effect on P concentration in soil solution.

A factorial analysis of variance showed a significant ($p < 0.01$) effect of genotype × Al interaction on root length (RL), specific root length (SRL), and shoot dry matter (SDM), but not on root dry matter (RDM). Non-linear regression was performed between total root length and lime rates for each genotype. The regression equations were then used to calculate the requested lime rates to reach 90% of the maximum total root length development, as often used to calculate critical values (Smith and Loneragan,

1997), and the genotypes were ranked for Al resistance according to their requested critical rate of lime.

Table 2. Analysis of soil solution from pots with plants (+ plants) and respective blanks without plants (− plants) after harvesting

Lime rate	pH	Al	P	K	Ca	Mg	DOC[1]
g $Ca(OH)_2$ kg^{-1} soil				μmol L^{-1}			mg L^{-1}
(− plants) 0.0	3.36	1434	13.2	5960	555	2185	467.5
(+ plants) 0.0	3.39	1278	14.8	4650	509	2219	476.5
(− plants) 0.5	3.63	492	14.8	6039	4222	1923	390.4
(+ plants) 0.5	3.63	389	15.2	1025	3035	1414	422.8
(− plants) 1.0	3.86	229	14.5	5718	7410	1888	360.6
(+ plants) 1.0	3.82	218	18.4	631	5011	1139	416.8
(− plants) 1.9	4.09	127	11.3	5417	15702	2289	328.3
(+ plants) 1.9	4.12	111	12.9	439	9309	988	383.0

[1] DOC: dissolved organic carbon

The growth of all genotypes was markedly reduced with increasing soil Al saturation. However, the maize genotypes differed widely in response to the addition of lime to soil (Table 3 and Figure 1). G4 and G5 showed a much faster increase in root length in response to addition of lime than G6, G8, and G9. Consequently, G4 and G5 reached 90% of their maximum root development within the range of conditions imposed, while G6, G8, and G9 did not. At the highest lime rate the root length produced as related to the maximum estimated using the equations was 64%, 57%, and 79% for G6, G8, and G9, respectively.

Apart from the reduction in root length caused by Al, its deleterious effects on the root system could also be seen on the specific root length (SRL) (Table 4). With unlimed soils, roots of all genotypes were shorter and thicker than roots of plants growing on limed soils, which is known as a typical symptom of Al-induced root damage. With the addition of 0.5 g $Ca(OH)_2$ kg^{-1} soil, the SRL increased substantially with all genotypes and the SRL values of 6 of them (G1, G2, G3, G4, G5, G7) were not significantly different from those grown at the highest lime rate. In contrast, the other 4 genotypes (G6, G8,

G9, G10) needed higher rates of lime to produce SRL values more similar to their roots growing under low Al stress conditions.

Table 3. Equation parameters and critical rates of lime to be applied for 90% of maximum total root length of maize genotypes grown in an acid soil

	RL = $a + b \times [1 - \exp(-c \times \text{lime})]$[1]				
Group/Genotype	**Parameters**				Critical Rate (**CR**)
	a	*b*	*c*	r^2	g $Ca(OH)_2$ kg^{-1} soil
I. Resistant					
G5	117.9	243.5	2.3432	0.89	0.81
G4	126.7	330.5	2.1673	0.85	0.91
Group Mean					0.86
II. Intermediate					
G1	144.4	384.8	1.2868	0.85	1.54
G7	97.4	223.4	1.2489	0.89	1.55
G2	81.3	278.8	1.2021	0.88	1.70
G10	103.4	332.4	1.1801	0.94	1.72
G3	98.6	479.0	1.1846	0.92	1.79
Group Mean					1.66
III. Sensitive					
G9	57.9	450.3	0.7580	0.90	2.88
G6	82.2	687.3	0.4804	0.95	4.56
G8	125.8	961.8	0.3802	0.94	5.73
Group Mean					4.39

[1] RL: total root length (m $plant^{-1}$); lime: rate of lime (g $Ca(OH)_2$ kg^{-1} soil)

Culture Solution Test

The analysis of variance showed significant differences between genotypes and a significant effect of Al on root length (RL), specific root length (SRL), shoot dry matter (SDM), and root dry matter (RDM). Al caused a general reduction of all plant charac-

teristics evaluated, but as reduction varied among genotypes, a significant effect of the interaction genotype × Al was found for RL, SRL, and SDM. The plant characteristics used to evaluate differences in Al resistance among the genotypes showed differential magnitudes of sensitivity to Al. Undoubtedly, RL showed the highest sensitivity to Al, followed immediately by SRL. The reductions in RL and SRL caused by Al were significant ($p < 0.01$) in all genotypes (Table 5) and the widest range of Al resistance, as expressed by the ratio [+Al/−Al], was observed using RL. Moreover, a significant difference between the most Al resistant genotypes (G4 and G5) and the most sensitive one (G9) was only detected when RL was used. Reductions in SDM and RDM were less drastic among the genotypes, although some of them were significant.

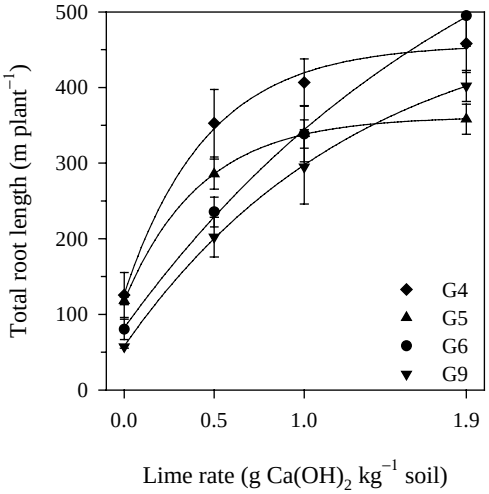

Figure 1. Total root length response of four maize genotypes differing in Al resistance (G4, G5, G6, and G9) to the application of increasing rates of lime to an acid soil. Vertical bars denote ± SE.

Results of a chemical test on root organic anion concentration, a possible indicator or biomarker, illustrating the plant resistance to Al showed significant genotype × Al interaction on root concentration of citrate and malate. Exposure to Al caused an overall increase in concentration of both organic anions in the roots (Figure 2). Root concentrations of citrate and malate were significantly higher in the presence of Al, with exception

of malate in G6, G8, and G9. The increase in root concentration varied from 91% (G6) to 196% (G7) for citrate and from 9% (G9) to 132% (G1) for malate. The genotypes differed significantly in root concentrations of both organic anions, but only in the presence of Al (Figure 2). Without Al in the rooting medium, the mean concentrations were 4.1 mmol kg^{-1} root dry matter for citrate and 14.3 mmol kg^{-1} root dry matter for malate. However, a grouping of the ten genotypes according to their root concentrations of citrate or malate (Table 5) did not coincide with the grouping according to their differential Al resistance (Tables 1, 3, 4, and 5).

Table 4. Specific root length of maize genotypes in response to the application of lime to an acid soil. Within each genotype, only values marked with ** ($p < 0.01$) or * ($p < 0.05$) are significantly different from those of the highest lime rate

Group/Genotype	Specific Root Length (m g^{-1} root dry matter)			
	Lime rate (g $Ca(OH)_2$ kg^{-1} soil)			
	0.0	0.5	1.0	1.9
I. Resistant				
G5	105.2*	137.5	150.6	133.8
G4	97.7**	155.1	142.2	137.1
II. Intermediate				
G1	97.6**	143.7	159.8	147.6
G7	96.5**	134.7	150.5	159.2
G2	103.2**	148.1	176.6	169.9
G10	90.3**	108.8**	158.1	156.3
G3	81.1**	126.4	151.0	144.3
III. Sensitive				
G9	80.0**	122.4**	155.4	166.5
G6	85.2**	134.3**	156.1	178.4
G8	81.9**	144.8**	131.2**	183.4

Discussion

Genetic variability with respect to Al resistance existed among the screened maize genotypes in an acid soil and in culture solution. The genotypes showed a consistent reaction to the stress factors Al or pH when grown in different rooting media under different stress intensities. Due to different root elongation rates (RER) of the genotypes, the actual Al resistance of a particular genotype is confounded when RER are compared. Thus, relative Al resistance in the root elongation assay was calculated within each genotype by comparing root growth under different Al concentrations with zero Al as control. These comparisons showed that the genotypes G7, G8, and G9 were much more affected by Al than G1, G4, and G5, the latter three classified as Al resistant. Most of the genotypes showed enhanced root elongation at 10 μM Al. Enhancement of growth by low concentrations of Al has been observed in maize (Llugany et al., 1995) and in other plant species (Bollard, 1983). The proposed mechanisms by which low Al concentrations may stimulate plant growth include improvement of Fe and P nutrition, alteration in the distribution of growth regulators, prevention of Cu and Mn toxicities (Foy, 1984), and alleviation of H^+ toxicity (Kinraide, 1993).

The seedling screening method based on RER was effective in separating resistance differences among genotypes and can be used as a reliable and early indicator of Al resistance in maize. However, as noted by Sartain and Kamprath (1978), short-term root elongation studies are probably only reflecting the effects of Al on root cell elongation and cell division whereas longer-term studies, either in nutrient solution or in acid soil, give an integrated effect of Al toxicity on plant growth, eventually including final grain yield.

A great concern when screening germplasm for Al resistance is that genotypes can interact with the screening medium, leading to false ratings and not showing all genetic sources of Al resistance. Also, the classification of genotypes based on their Al resistance in rapid screening methods very often does not correlate well with their growth response in acid soils (Kasim et al., 1990). However, in the current study a good agreement was found on the performance of the genotypes. They were ranked for Al resistance in the same general order both in culture solution and in soil, although genotypes 1 and 7 showed small discrepancies across the rankings. Classified as Al resistant in the root elongation assay, G1 exhibited an intermediate performance in the acid soil and in the culture solution test where possibly factors other than Al may have caused it a lower performance. On the other hand, G7, classified as Al sensitive in the root elongation assay showed a response to lime that conferred it a high position in the intermediate group. Higher resistance to H^+ toxicity and higher efficiency in uptake of P, Ca, and Mg under acid conditions are among the possible reasons that may have conferred G7 a better per-

formance. As found for sorghum genotypes grown in the presence and absence of toxic concentrations of Al, the greater potential for increasing dry matter yields of Al resistant genotypes could be partly because they had higher root influx rates of some nutrients (Baligar et al., 1993).

Table 5. Effect of the addition of 100 μM Al (relative to 0 μM Al) on total root length (**RL**), specific root length (**SRL**), shoot dry matter (**SDM**), root dry matter (**RDM**), root concentration of citrate (**CIT**), and root concentration of malate (**MAL**) with plants of ten maize genotypes grown for 14 days in culture solution. Relative values of the various plant characteristics are expressed by [(+Al/−Al)×100]. Values marked with ** ($p < 0.01$) or * ($p < 0.05$) indicate significant reduction caused by Al. Within each plant characteristic, values followed by a different letter are significantly different ($p < 0.05$)

Group/Genotype	**RL**	**SRL**	**SDM**	**RDM**	**CIT**	**MAL**
I. Resistant						
G4	60** a	63** a	80* a	107 a	260** a	195** a
G8	52** a	61** a	60** a	69** a	274** a	143* a
G5	51** a	58** a	81* a	84 a	195** a	181** a
II. Intermediate						
G3	47** ab	56** a	61** a	87 a	265** a	208** a
G6	46** ab	48** a	70** a	83 a	191** a	132 a
G1	44** ab	47** a	68** a	77* a	262** a	232** a
G7	40** ab	44** a	57** a	71** a	296** a	184** a
G2	39** ab	52** a	61** a	70** a	n.d.[1]	n.d.
G10	38** ab	43** a	62** a	73* a	211** a	148* a
III. Sensitive						
G9	26** b	41** a	62** a	65** a	265** a	109 a

[1] n.d.: not determined

It seems that 100 μM Al in the culture solution test, in combination with the time of exposition to Al (14 days), had a too severe effect on root development of the genotypes, making them less resistant to Al. Nevertheless, the Al resistance ranking obtained is in

agreement with those obtained in the other two experiments described. Most of the genotypes with an intermediate resistance to Al stayed in such a group. Ranking the genotypes based on RL and SRL, conferred G8 a better position than previously. However, in terms of shoot biomass this genotype had the second highest reduction among all the others. G5 and G4, classified as Al resistant, performed significantly better than did the Al sensitive G9 (Table 5).

Difference in Al resistance of a genotype as observed in experiments with acid soils and culture solution might also be partly due to different availability of nutrients and water in the two different rooting media. Moreover, in this work Al concentrations in the culture solution were much lower than those found in soil solution of the acid soil as used in the soil experiment. That even at much higher Al concentrations in soil solution effects of Al in soil and culture solution were comparable has to be explained by differences in Al speciation among the two substrates. In the soil as used, concentrations of dissolved organic carbon (DOC) were high, probably leading to a partial complexation and detoxification of the soluble Al with organic anions as citrate and fulvate (Hue et al., 1986; Suthipradit et al., 1990). Just contrary, in culture solution most of the Al will be present in the toxic form as monomeric Al (Kinraide, 1991). Furthermore, in soils the rhizosphere established confers local conditions that are different from the bulk soil, mainly with respect to pH, and Al and root exudates concentrations (Marschner, 1991). This often frustrates comparison of experiments with nutrient solution and soil as rooting substrate.

Root length characteristics have been preferred as indicator of quantitative evaluation of plant resistance to Al toxicity over root dry matter and shoot characteristics, especially in short-term experiments, when most pronounced effects of Al take place in the root system. When using root dry matter, no significant interaction effect of genotype and Al was found in the soil and culture solution test. A strong effect of Al on root morphology was shown (Tables 4 and 5). Under Al stress conditions, most genotypes showed low values of SRL indicating a root system with short roots of increased diameter and poor branching. The lateral root initiation and development are strongly influenced by environmental factors, including Al toxicity, and it is generally accepted that the total length of a root system is mainly determined by the length of fine branch roots (de Willigen and van Noordwijk, 1987). Furthermore, lateral roots provide important means of constructing a root system, increasing its absorptive area and the volume of substrate exploited. Therefore, a poorly developed root system will show a range of secondary effects of Al such as disturbance of hormone balance, deficiency of essential nutrients, inhibition of photosynthesis, alterations in carbon allocation, and alterations in water relations (Foy, 1984). All these changes are partly caused by an inhibition of root growth and finally result in plant growth reduction.

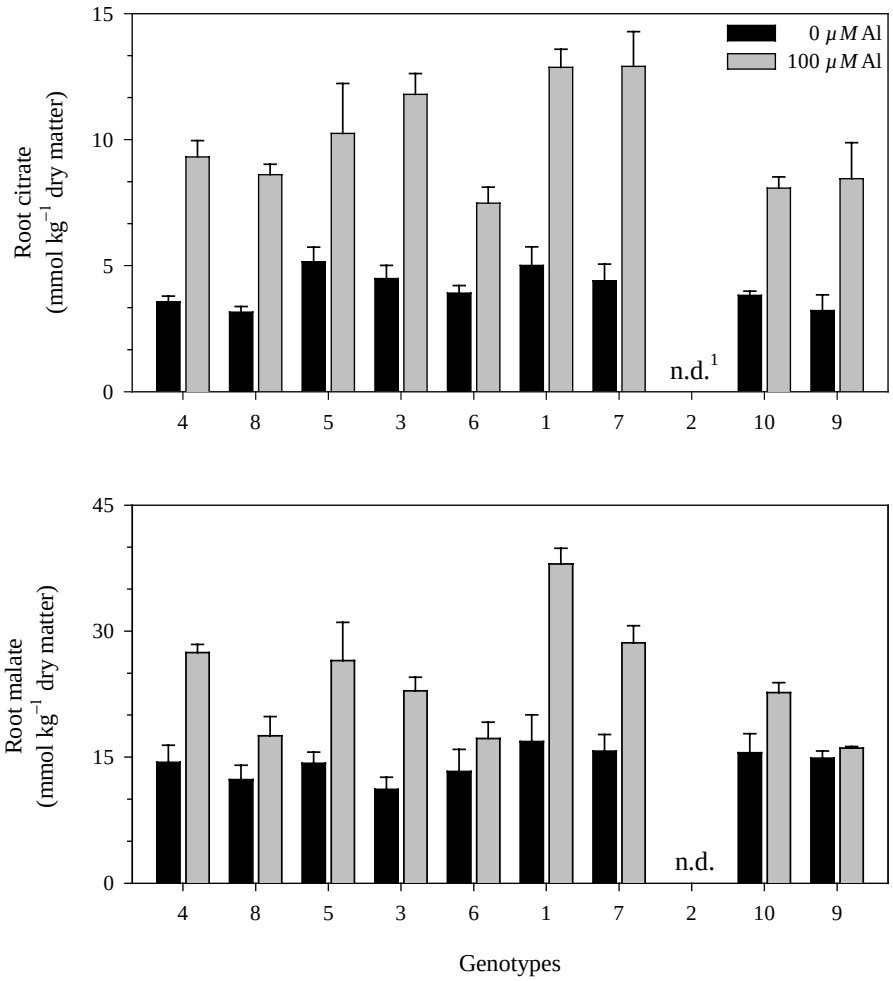

Figure 2. Organic anion concentration in roots of maize genotypes growing at two concentrations of Al in solution. Data are means of three replicates ± SE. [1] n.d.: not determined.

The critical rates of lime application calculated in the soil experiment indicated how genotypes reacted to the application of lime and alleviation of soil acidity. These critical rates can be interpreted as the level of soil acidity or pH requested by genotypes for op-

timal root development. While lime application rates higher than 1.0 g $Ca(OH)_2$ kg^{-1} soil did not cause any further significant increase in root length with genotypes G4 and G5 of the resistant group I, the genotypes of group III were still responding significantly to the alleviation of soil acidity at such lime rate, suggesting that the plants were still experiencing Al toxicity. The potential root development of Al sensitive genotypes was probably not fully realised even at the highest lime rate since the calculated doses of lime to achieve 90% of their maximum root length were beyond the range studied.

Results of a test to use the internal root concentrations of organic anions, particularly that of citrate, as a biomarker that might reflect plant resistance to Al were not promising. The presence of Al in the rooting medium significantly increased internal root concentrations of citrate and malate with most genotypes, but genotypic variation in root concentration of these organic anions could not explain the differences in resistance to Al as observed in our work. An increase in the concentration of citrate and malate in roots exposed to Al has been reported in maize (Gaume et al., 2001; Pintro et al., 1997), sorghum (Galvez et al., 1991), and wheat (Foy et al., 1990). Studying pairs of maize cultivars contrasting in Al resistance, Gaume et al. (2001) and Pintro et al. (1997) found that the increase in concentration of organic anions in roots was higher in the Al resistant cultivar than in the Al sensitive one, suggesting that the increase in concentration of organic anions may contribute to Al resistance. However, changes in organic anion concentration as found in our work have to be interpreted as the result of Al-induced stress and not as a basis for differential Al resistance. Results of our work also agree with those of Foy et al. (1990) who found no correlation between differential Al resistance of five wheat cultivars and changes in organic anion concentration in either shoots or roots.

The results of the screening techniques were used jointly to enhance the identification of Al resistance and the most contrasting genotypes were well defined. G4 and G5 were very resistant to the stress conditions tested. Genotype 5 has consistently shown better growth responses than other genotypes when grown under Al stress (Kasim and Wassom, 1990; Lopes et al., 1987) and is known to be resistant to acid soils (Pandey et al., 1994). Lopes et al. (1987) reported that G5, together with the experimental hybrid CMS200, showed the lowest reduction (27%) in relative elongation of the seminal root after 8 days at 222 μM Al in a test study for Al resistance involving 13 maize populations. Genotype 5 was developed from lines with superior performance in tropical acid soils and was indicated as source of resistance to Al in breeding programs. G9 showed to be very sensitive to Al, being classified as Al sensitive in all the experiments performed. G5 and G9 are therefore good candidates for further study of Al resistance mechanisms, as described in Chapter 3.

In conclusion, the three screening techniques used produced very similar results, leading to a consistent Al resistance ranking of the genotypes. In contrast, internal root

concentration of organic anions could not be used as indicator of Al resistance. Al resistance in relation to plant organic anions and their exudation by roots will be studied in Chapter 3, where aspects of organic anion exudation by roots in response to Al and their possible role in resistance to Al will be the main topic.

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Chapter 3

Evaluating the role of root citrate exudation as a mechanism of aluminium resistance in maize genotypes[♣]

Abstract – Organic anion exudation by roots as a mechanism of aluminium (Al) resistance has been intensively studied lately. In the present study we evaluated qualitative and quantitative aspects of root exudation of organic anions in maize genotypes of distinct sensitivity to Al in response to Al exposure. Roots of maize seedlings were grown axenically in nutrient solution and root exudates were collected along the whole seminal root axis for a short period (4 h) using a divided-root-chamber technique. In root exudates collected from 10-mm long root apices, citrate accounted for 67% of the total organic anions found, followed by malate (29%), *trans*-aconitate (3%), fumarate (<1%), and *cis*-aconitate (1%). Rates of citrate exudation from root apices of two genotypes with differential resistance to Al were consistently higher in the Al resistant one, differing by a factor of 1.7–3.0 across a range of external Al concentrations. Furthermore, relative Al resistance of eight maize genotypes correlated significantly well with their citrate exudation rate measured at 40 μM Al. Higher exudation rates were accompanied by a less inhibited root elongation. The exudation of citrate along the longitudinal axis of fully developed seminal roots showed a particular pattern: citrate was exuded mainly in the regions of root apices, either belonging to the main root or to the lateral roots in the most basal part of the main root. The involvement of citrate in a mechanism of Al resistance is evaluated in terms of protection of the root from the effects of excess Al on root elongation and on nutrient uptake along a root axis showing distinct sites of citrate exudation.

Key words: aluminium exclusion, aluminium toxicity, citrate, organic anions, sterile root exudates, *Zea mays* cultivars

[♣] with Willem G. Keltjens

Introduction

Aluminium (Al) toxicity is a major factor limiting plant growth on acid soils. Strategies to overcome the negative effects of Al on plant growth in these soils include the application of lime to raise soil pH and lower soil exchangeable Al, and the use of crop species that are resistant to Al-toxic soils (Foy, 1984). Species like maize, sorghum, soybean, and wheat show considerable intraspecific variation in resistance to Al. Plant breeders have exploited this variation in maize aiming to develop genotypes with superior resistance to Al and soil acidity conditions (Pandey et al., 1994). Differences in varietal resistance to Al have been considered in terms of mechanisms facilitating Al exclusion from the root symplast (Al exclusion) and mechanisms conferring the ability of plants to tolerate Al in the plant symplast (Al tolerance) (Kochian, 1995).

Exclusion mechanisms are those mechanisms where metals are prevented from crossing the plasma membrane, entering the symplast, and reaching sensitive intracellular sites. Exclusion mechanisms might include immobilisation at the cell wall, selective permeability of the plasma membrane, a plant-induced pH barrier in the rhizosphere, exudation of chelate ligands, exudation of phosphate, and Al efflux (Taylor, 1991). The exudation of metal chelators into the root apoplast in response to Al toxicity has received much attention during the last years. In this hypothesised mechanism, the compounds released by root cells into the apoplast would form complexes with Al, thus reducing the Al^{3+} activity locally and reducing Al absorption across the plasma membrane. Reduction of Al^{3+} activity in the apoplastic compartment itself would also confer Al resistance, since Al interactions with the cell wall and cell membranes, preceding any transport into the symplast, are potentially harmful (Delhaize and Ryan, 1995).

Among the potential metal chelators released by roots, a wide range of organic compounds has been proposed. They vary from root mucilage, which actually is a complex mixture of root cap mucilage, metabolically active root border cells, and cell wall fragments (Miyasaka and Hawes, 2001), to low-molecular-weight organic anions (Ryan et al., 2001). Low-molecular-weight organic anions such as citrate, fumarate, malate, and oxalate have been intensively studied as possibly involved in a mechanism of Al resistance in plants, due to their involvement in many metabolic processes and to their negative charge, conferring them the capacity to complex metals. It was proposed that the exudation of malate from root apices could protect the root by chelating and thus detoxifying Al in the rhizosphere (Delhaize et al., 1993). Studying a pair of near-isogenic wheat lines that differ in Al resistance at a single dominant locus, Delhaize et al. (1993) found a good correlation between Al-induced malate release, Al resistance, and Al exclusion from the root apex. Root exudation of organic anions as a mechanism of Al ex-

clusion has also been supported by studies with other species like buckwheat (Zheng et al., 1998), maize (Jorge and Arruda, 1997; Pellet et al., 1995), snapbean (Miyasaka et al., 1991), and soybean (Silva et al., 2001; Yang et al., 2000). Specifically with maize, exposure to Al^{3+} induced a rapid release of mostly citrate and malate by roots of genotypes showing differential sensitivity to Al (Gaume et al., 2001; Kollmeier et al., 2001; Pellet et al., 1995). Each of these studies evaluated a pair of genotypes of significant differential sensitivity to Al. Because higher exudation rates were observed in the more Al resistant genotype of each pair, the authors generally proposed that this mechanism of protection against toxic Al might be also operative in maize. However, a relationship between resistance to Al and organic anion exudation within a group of genotypes with distinct degrees of Al resistance has not yet been shown.

In most of these studies root exudates were either collected only from the apical region of roots (Kollmeier et al., 2001), or from the entire root system with the assumption that the measured exudation has occurred solely in the root apex (Gaume et al., 2001; Jorge and Arruda, 1997). In both cases, exudation only from the root apex or exudation from the entire root, no information is made available on the spatial or longitudinal distribution of organic anion release along the root. Although it is now well established that Al causes reduction in root growth only when in contact with the apical 2–3 mm of the root (Ryan et al., 1993; Sivaguru and Horst, 1998), one could speculate that release of organic anions exclusively at the root apex would only protect this part against excess Al, but would leave the rest of the root unprotected against the well-known negative effects of Al on nutrient uptake (Keltjens, 1995; Rengel and Robinson, 1989a).

There are few studies that investigated exudation by root zones other than the apical one (Pellet et al., 1995; Piñeros et al., 2002). Although they both used maize seedlings in their experiments, these studies showed distinct results. Pellet et al. (1995) reported that neither citrate nor malate exudation was measured in mature zones of the root. Conversely Piñeros et al. (2002) reported recently that root segments as far as 60 mm behind the root tip can exude citrate at rates very much similar to those measured within the first mm of the root tip. Because they used in their studies methodologies that are basically different [intact roots in Pellet et al. (1995) versus excised root segments in Piñeros et al. (2002)] a fair comparison of the results seems impossible. Furthermore the pattern of organic anion exudation along the root axis cannot be concluded from these conflicting results. To resolve this question and to get more insight into the longitudinal distribution of organic anion release along intact, whole root systems of maize seedlings as well as into other aspects of the exudation of organic anions on Al resistance in maize this study was established.

The aims of the present work were therefore to study: (*i*) the effect of Al exposure of maize genotypes differing in Al sensitivity on root organic anion exudation, both quan-

titatively and qualitatively, (*ii*) the relationship between Al resistance and the root organic anion exudation rate, and (*iii*) the spatial distribution of organic anion release along intact roots of maize seedlings. In this paper, a first qualitative evaluation will be made on the possible role of organic anions in a resistance mechanism to Al. The role of citrate will be evaluated in terms of protection of the root from the effects of Al on root elongation and on nutrient uptake along a root axis showing distinct sites of citrate exudation.

Materials and methods

Plant material and seedling growth

Two genotypes of maize (*Zea mays* L.) showing differential resistance to Al were intensively studied, while behaviour of six other genotypes was studied only in one experiment. All eight genotypes were selected from a collection of ten Brazilian maize genotypes screened before for resistance to Al in both culture solution and acid soil. The two genotypes, CMS36 (Al resistant) and BR106 (Al sensitive), showed the greatest difference in resistance to Al among the ten genotypes tested (Chapter 2).

Roots of seedlings were grown axenically to prevent microbial degradation of organic compounds exuded by roots. All materials used in the experiments were autoclaved and successive treatments were carried out in a laminar-flow hood. To sterilise the surface of the seeds, they were immersed for 1 min in 96% ethanol, soaked for 1 h in a solution containing 1.5% sodium hypochlorite (from commercial bleach) + 1% Tween 20, and subsequently incubated for 15 min in a 1.5% sodium hypochlorite solution. After each treatment the seeds were rinsed three times with sterile demineralised water. To check for eventual microbial contamination surface-sterilised seeds were germinated on nutrient agar plates prepared with a 1 m*M* $CaSO_4$ solution. The plates were placed in a dark chamber at 25 °C for 90 h. After germination, uncontaminated seedlings were individually transferred to Petri dishes (∅ 94 mm) containing 50 mL of a sterile basal nutrient solution with pH 4.0 and composition (m*M*): 1.0 NH_4NO_3, 0.005 NaH_2PO_4, 0.5 K_2SO_4, 0.5 $CaCl_2$, 0.125 $MgSO_4$, and (μM): 46 B, 0.3 Cu, 286 Fe (as Fe-EDTA), 0.1 Mo, 9.2 Mn, 0.8 Zn. The nutrient solution was adjusted to pH 4.0 with 0.1 *M* HCl and autoclaved before the addition of filter-sterilised Fe-EDTA stock solution.

In each Petri dish, the roots of one seedling were grown horizontally oriented whereas the shoot was grown vertically through a small notch made in the edge of the lid. The notch was sealed with lanolin and the Petri dish wrapped with foil and transferred to a growth chamber where the seedlings were grown for 3 days at 20 °C under a regime of 16 h light (light intensity 80 W m^{-2})/8 h dark.

Aluminium treatments and collection of root exudates

Aluminium was added to the sterile basal nutrient solution with composition as described above (without Fe-EDTA) to reach concentrations ranging from 0 to 100 μM Al. Al was diluted from a filter-sterilised stock solution containing 10 mM $AlCl_3$ and 0.1 mM HCl. After the 3-d pre-growth, the Al treatment solutions (50 mL) were applied to the seedlings in the same Petri dishes in which they had been growing during the preceding 3 days, replacing the old nutrient solutions. The time of exposure to Al varied from 0 to 24 h before the collection of root exudates started.

The divided-root-chamber technique, described by Ryan et al. (1993) and Delhaize et al. (1993), was used to study the exudation of organic anions by roots of maize seedlings in response to Al in solution. Organic anion exudation was studied not only over the apical region of roots, but also along the longitudinal axis of the whole main root of the seedlings. After 0 to 24 h of Al pre-treatment, seedlings were transferred to large Petri dishes (∅ 145 mm) where they had their roots spatially divided using plastic rings (∅ 13 mm). These plastic rings were placed over the apical 10 mm of every seminal root of a seedling (Figure 1A) or over the whole main root only (Figure 1B), covering it all from the root apex to the most basal section of the root. A thin layer of vaseline was used to seal the space between the plastic ring and the bottom of the Petri dish and a layer of agar was poured around each plastic ring to hold it over the root. Each plastic ring isolated either an apical 10-mm or a 13-mm long root section from the rest of the root system forming an individual chamber. An aliquot of 0.5 mL of Al treatment solution was applied to each chamber and with 50 mL of the same treatment solution the rest of the root system was covered. The Petri dish holding the system was closed with a lid to prevent contamination and evaporation of the treatment solutions, wrapped with foil, and transferred to the growth chamber. The solutions enclosed by the rings (diluted root exudates) were collected after 4 h and stored at −18 °C. An 8-μL aliquot of the diluted root exudate was plated on nutrient agar and incubated in a dark chamber at 25 °C for 7 days to check for eventual microbial contamination. Contaminated root exudates were discarded.

To study the effect of time of exposure to Al prior to the organic anion exudation measurement, seedlings of the Al resistant genotype were incubated for 30 h in a 40 μM Al solution. In this time interval, the root exudates were collected twice, i.e. during the intervals [4-10]h and [24-30]h after the start of the exposure.

Root exudation of organic anions in response to Al concentrations in solution (0, 10, 20, 40, 100 μM Al) was studied in the root apices of both genotypes. Each Al concentration was tested in 3–4 seedlings of CMS36 and 5 seedlings of BR106. Within each seed-

ling, 2–6 root apices were sampled in CMS36 and 3–6 apices in BR106. Root exudates were collected after 24 h of pre-treatment at corresponding Al concentrations.

The distribution of organic anion exudation along the longitudinal axis of the main root was studied only in the Al resistant genotype in an experiment with two Al concentrations (0 and 40 μM Al). Root exudates were collected after 24 h of pre-treatment at 0 or 40 μM Al. At the end of the experiment the number of lateral roots enclosed by each plastic ring on the more basal section of the root was counted under a binocular (3.2 × magnification). Three to five replicate seedlings were prepared for each treatment and the entire experiment was repeated once. Because the seedlings used did not have roots of the same length, the number of plastic rings used to cover the entire root varied among the replicates. Hence, the exudation values measured in the plastic rings could not be directly compared between replicates, unless the number of rings used was the same. To overcome this problem, we considered each plastic ring as representing a percentage of the total length of the root. Therefore, the total root length was divided in 100 units (= 100%) and the citrate exudation value of each ring was repeatedly attributed to every unit that such ring represented. Exemplifying, for a seedling in which nine rings were used to cover the whole root, each ring represented eleven units (= 11%) and the nine rings completed 100 units. Once every root had the same relative length, the average of every unit (1%) was calculated among the replicates of the treatments.

Besides CMS36 and BR106, six other genotypes (64×1143, HD91102, 13×1143, 11×723, HD9148, and 20×22) were used in a series of experiments in an attempt to find a relationship between Al resistance and root exudation of organic anions. For this purpose, root exudates were collected from the root apex of the main root of seedlings of all genotypes (five seedlings of each genotype) after a pre-treatment at 40 μM Al for 24 h. Data on the Al resistance of these genotypes were obtained in a previous work (Chapter 2). The Al resistance of the genotypes was expressed by the percentage reduction in the root elongation rate caused by 40 and 100 μM Al, relative to root elongation rate of seedlings grown in control solution (0 μM Al).

Analysis of organic anions in root exudates

To get information about the distribution of various types of organic anions exuded, root exudates of the first experiment were first analysed by reversed phase High Performance Liquid Chromatography (HPLC) in the ion suppression mode. Separation was conducted on a 250 × 4 mm reversed phase column (GROM-SIL 120 ODS-3 CP, 5 μm particle size) equipped with a 20 × 4 mm Hypersil ODS guard column (Grom, Herrenberg, Germany). Sample solutions (20 μL) were injected onto the column, and 18 mM KH_2PO_4 adjusted to pH 2.1 with H_3PO_4, was used for isocratic elution, with a flow rate of 0.5 mL min^{-1} at 28 °C and UV detection at 215 nm. Identification of organic anions was per-

formed by comparing retention times and absorption spectra with those of known standards (Neumann and Römheld, 1999).

The diluted root exudates collected from each plastic ring in all experiments were individually assayed for citrate. By using enzymatic methods, 0.66 mL of sample (0.50 mL of root exudate + 0.16 mL of water) was incubated with 0.33 mL of buffer (Glycylglycine, 0.51 mol L^{-1}; Zn^{2+}, 0.6 mmol L^{-1}; pH 7.8), 8 μL of NADH (6 mmol L^{-1}), and 7 μL of a mixture of malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) (0.5 mg MDH mL^{-1}; 2.5 mg LDH mL^{-1}). Oxidation of NADH, triggered by the addition of 7 μL of citrate lyase (40 U mL^{-1}), was monitored by UV spectroscopy at 340 nm on a chart recorder and is directly proportional to the amount of citrate in the sample (Boehringer Mannheim, 1984).

Statistical analyses

A two-factor nested ANOVA was applied for each genotype in the experiment of exudation of organic anions in response to Al concentrations to ascertain the magnitude of error at various stages of the experiment. The two factors were: (A) Al concentrations and (B) seedlings. The factor B (seedlings) is nested within factor A (Al concentrations) and used as an error term when testing factor A. The basic error variance is the variance of the apices measurements, used to test factor B. Non-linear equations were fitted to data on the relationship between Al resistance and root exudation of organic anions. The analyses were computed using the SAS software (SAS Institute, 1990).

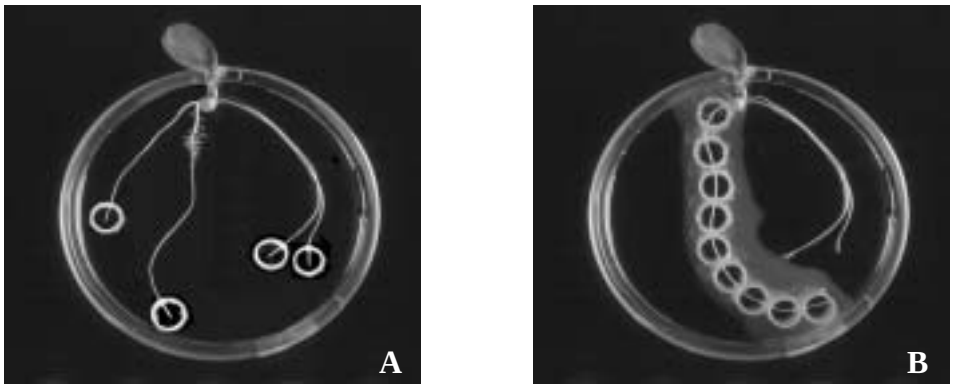

Figure 1. Aspects of the divided-root-chamber technique showing the plastic rings over (A) the root apices and over (B) the whole main seminal axis of a root system growing axenically in a Petri dish.

Results

Types of organic anions exuded

The HPLC analysis of root exudates collected from root apices of the Al resistant genotype grown at 40 μM Al detected citrate and malate as the major carboxylic anions exuded under the conditions imposed (Table 1). Together they accounted for 96% (67% of citrate and 29% of malate) of the total organic anions found, followed by much lower concentrations of *trans*-aconitate, fumarate, and *cis*-aconitate. Therefore, due to the dominance of citrate over the other organic anions and its high capacity to complex Al, in the subsequent experiments we limited our analyses to citrate, which was measured in root exudates by using enzymatic methods.

Table 1. Exudation rates of different organic anions from root apices of the Al resistant maize genotype CMS36 grown at 40 μM Al. Values are means ± standard errors of 6 replicates

Genotype	Exudation rate (pmol root apex^{-1} hour^{-1})					
	Citrate	Malate	*trans*-Aconitate	Fumarate	*cis*-Aconitate	total of organic anions
CMS36	256.5 ±59.9	110.0 ±29.8	10.4 ±3.1	1.8 ±0.4	2.0 ±0.7	380.7 ±77.5

Exudation of citrate by root apices

Root citrate exudation rate was significantly affected by the time that roots were exposed to Al prior to exudation measurement. The rate of citrate exudation was even doubled from the first to the second 6-h collection period, starting 4 and 24 h after Al exposure, respectively (Figure 2). Based on this strong increase of the citrate exudation rate with time of incubation to Al, in succeeding experiments root exudates were always collected after 24 h of pre-treatment at a given Al concentration. A pre-treatment with Al for a period longer than 24 h was avoided, because of direct effects of Al on root elongation during such a long time.

The effect of external concentrations of Al on the exudation of citrate was compared in root apices of both genotypes CMS36 and BR106. The addition of as little as 10 μM Al to the nutrient solution significantly stimulated the exudation of citrate with both genotypes (Figure 3). The exudation rates of citrate were distinct across the Al concentrations and genotypes studied. In the presence of Al, exudation rates by the Al resistant

genotype CMS36 were largely dependent on external Al concentration, and at least 70% higher than those found with the Al sensitive BR106. The biggest difference in exudation rates was observed at 40 μM Al, when root apices of the Al resistant CMS36 exuded three times the amount exuded by the Al sensitive BR106. However, in the absence of Al, citrate exudation rates were lowest and equal for both genotypes. The Nested ANOVA for the effect of Al on the exudation rates of citrate showed a more pronounced effect of Al with the Al resistant genotype, where the Al treatments had a highly significant effect on the exudation rates of citrate, being responsible for 57% of the total variation found. Seedlings and root apices added similar variations, 20% and 24%, respectively (Table 2). In the Al sensitive genotype, the concentration of Al had a less significant effect, and root apices added the largest part of the variation found (51%).

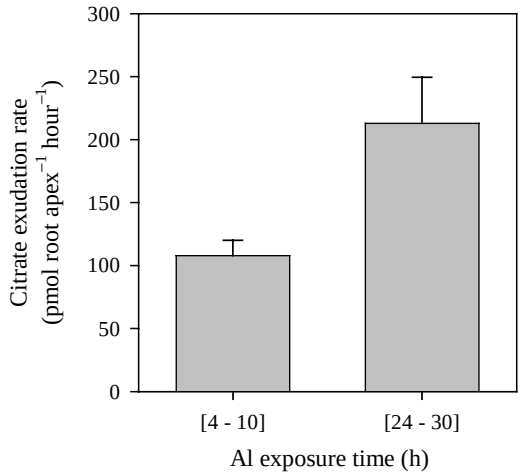

Figure 2. Exudation rates of citrate during the initial 30 h of exposure to 40 μM Al for the Al resistant maize genotype. The cumulative rate of citrate exudation of each collection period is presented as an average of 6 h of root exudate collection. Data show the means ± standard errors of 5 measurements.

Al resistance of the eight Brazilian maize genotypes as related to exudation of citrate is depicted in Figure 4. The exudation rates of citrate at 40 μM Al were plotted against the percentage reduction in the root elongation rate caused by 40 and 100 μM Al as found before (Chapter 2). These relationships were described by exponential equations: $y_{40} = 157.4 \times \exp(-0.0144x)$ ($r^2 = 0.40$; $P = 0.094$), and $y_{100} = 34.9 + 189.4 \times$

exp(−0.0186x) (r^2 = 0.70; *P* = 0.048), y_{40} and y_{100} representing root elongation rate inhibition (%) at Al concentrations of 40 and 100 μM, as dependent variables of the citrate exudation rate (pmol root apex^{-1} hour^{-1}). The genotypes CMS36 and BR106 that showed the lowest and highest inhibition in root elongation after Al exposure, and were qualified as Al resistant and Al sensitive, showed respectively the highest and one of the lowest citrate exudation rate at 40 μM Al.

Exudation of citrate along intact roots

The main roots studied were fully developed roots, about 120 mm in length and presenting short (< 5–6 mm) lateral roots in the more mature part of the root. This part of the root represented about 30% of the total length. The remaining of the root consisted of the root apex and of an intermediate zone, the part between the root apex and the zone with laterals. The apical zone of the root, enclosed by one plastic ring, represented about 15% and the intermediate zone about 55% of the total root length.

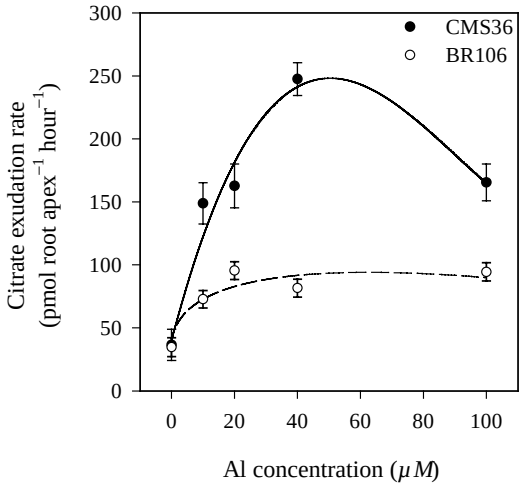

Figure 3. Effect of the external Al concentration on the exudation rate of citrate by root apices of the two maize genotypes showing the biggest difference in resistance to Al. Vertical bars denote ± SE.

Aluminium had a discernible and distinct effect on the exudation of citrate along intact roots of maize (Figure 5). Little citrate was released along roots of seedlings grown under control conditions (0 μM Al), whereas a clear pattern of exudation was observed

through the different morphological zones of roots at 40 μM Al. The highest exudation rates were observed in the apical region, a sharp decrease in the direction of basal zones, and a rise again in the zone containing lateral roots. The sites of highest exudation were therefore closely associated with the presence of root apices, either belonging to the main root or to lateral roots.

Table 2. Nested ANOVA for the data on citrate exudation in response to Al concentrations presented in Figure 3

CMS36					
Source	df	MS	F ratio	Pr > F	% [1]
Al concentrations (groups)	4	96283.4	8.45	0.0014	56.5
Seedlings (subgroups)[2]	13	11391.2	4.21	<.0001	19.9
Apices (error)[3]	52	2704.1			23.6
BR106					
Source	df	MS	F ratio	Pr > F	%
Al concentrations (groups)	4	11649.3	3.60	0.0228	21.6
Seedlings (subgroups)	20	3233.1	3.16	0.0001	27.1
Apices (error)	78	1024.7			51.3

[1] percentage of the total variation added by each source within each genotype
[2] variation among seedlings
[3] variation among apices of each seedling

Discussion

In our experiments, root exudates were collected from seedlings growing free from microorganisms and for short periods soon after the exudation of organic anions had been induced to higher rates. Dividing intact roots spatially in 13-mm segments (10-mm for the root tip) allowed not only a qualitative evaluation of sites of organic anion exudation but also a quantification of the fluxes of such organic molecules along the main root axis. This experimental set-up allowed us to determine accurately the exudation of organic anions during a period that root growth was minimum and re-absorption of exuded organic anions by the roots can be neglected (Jones and Darrah, 1995).

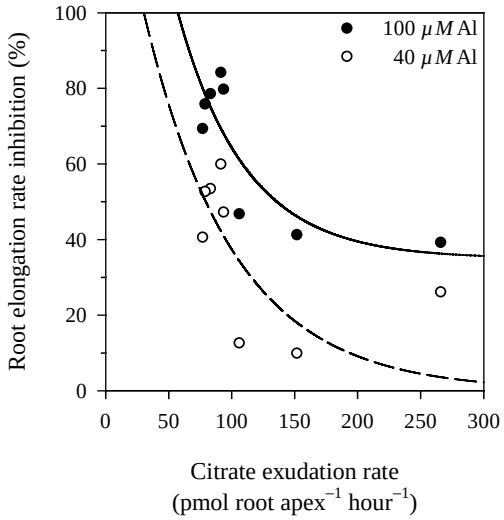

Figure 4. Relationship between exudation rates of citrate and root elongation rate inhibition of maize seedlings exposed to two concentrations of Al. Each data point represents a different genotype of maize. Root elongation rate inhibition as caused by 40 or 100 μM Al was calculated in reference to roots growing in solution without Al (0 μM Al). Values are means of 12 replicates. Citrate exudation was measured in root apices of seedlings exposed to 40 μM Al and values are means of 5 replicates. From the left to the right on the X-axis, the exudation rates correspond respectively to the genotypes: HD91102, 20×22, HD9148, BR106, 11×723, 64×1143, 13×1143, and CMS36.

Five organic anions were found in root exudates of the Al resistant maize genotype under conditions of Al stress. However, only the exudation of citrate and malate, representing the majority of the organic anions exuded, seems to be the result of certain physiological processes initiated or activated in response to Al toxicity. This finding is consistent with recent reports on root release of citrate and malate induced by toxic Al in maize (Jorge and Arruda, 1997; Kollmeier et al., 2001) as well as in other plant species (for a review see Ryan et al., 2001). According to literature organic anions of low molecular weight are respectively released into root apoplast and rhizosphere in response to a number of well-defined environmental stresses (e.g. Al toxicity, and P and Fe deficiency) and these responses are highly stress- and plant species-specific (Jones, 1998). Citrate and malate are well-known strong metal chelators. They have a high affinity for

trivalent metals such as Al^{3+} and Fe^{3+} and their effectiveness in reducing Al^{3+} activity and in alleviating the toxic effects of Al^{3+} to plants has been demonstrated in diverse circumstances (Hue et al., 1986; Keltjens, 1995; Ownby and Popham, 1989).

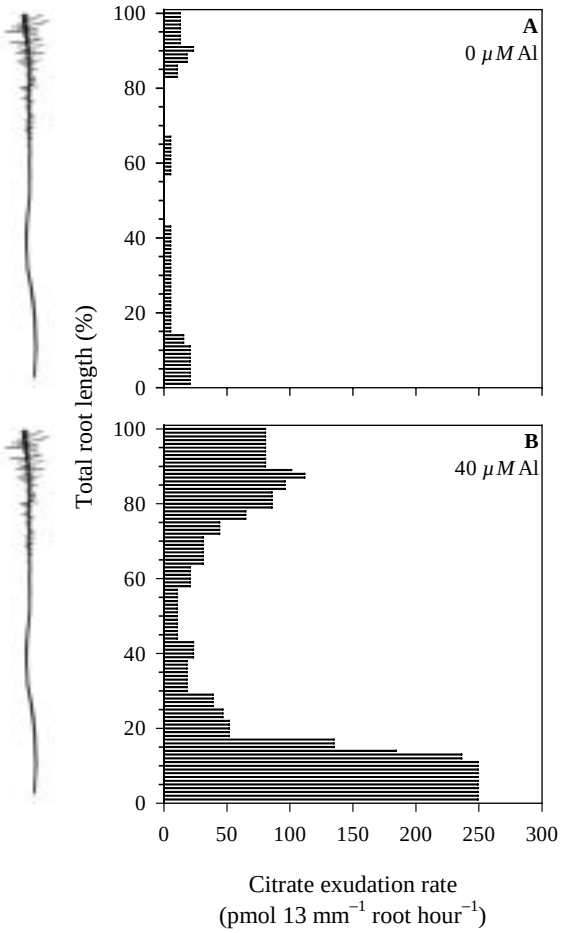

Figure 5. Exudation rates of citrate along the main root axis of the Al resistant maize genotype CMS36 exposed to 0 (A) or 40 μM Al (B). On the Y-axis 100 values are plotted. They represent the average exudation rates along the root axis of 6 replicates calculated as explained in Materials and methods. Root axes as used in the experiments are schematised on the left-hand side for reference.

Increased rate of citrate exudation with increased time of pre-treatment assumes the existence of an induction period, probably including an initial phase during which Al has to enter the root, followed by an Al-induced increase in citrate production, possibly combined with an increase in root cell permeability (Kollmeier et al., 2001). This corresponds with the findings that citrate exudation rates increased with increasing external Al concentration. However, if exceeding a certain concentration (40 μM Al), citrate exudation decreases again with a further increase in Al concentration. At too high concentrations, Al seems to become phytotoxic and to disturb certain biochemical processes involved in citrate synthesis. This automatically implies that resistance mechanisms based on root organic anion exudation, as described in this chapter, have their limitation what resistance to soil acidity or Al toxicity concerns.

Root apices of the main (longest) and the secondary seminal roots exuded citrate similarly under the experimental conditions tested, suggesting they all possess the mechanism controlling citrate release. Higher citrate exudation rates in the Al resistant maize genotype CMS36 were closely related to its higher resistance to Al. For example, at 40 μM Al, the concentration that caused the greatest difference in citrate exudation between the two genotypes (Figure 3), the Al resistant genotype had a reduction of 26% in root elongation rate (RER), while the Al sensitive one had a reduction of 60% in RER (Chapter 2). Higher exudation rates of citrate and malate have consistently been found in Al resistant genotypes of maize when pairs of genotypes representing extremes of Al sensitivity are compared (Gaume et al., 2001; Jorge and Arruda, 1997; Kollmeier et al., 2001). Thus, exudation of citrate or malate, depending on the plant species, has been suggested to correlate with resistance to Al. However, attempts to show such relationship in a greater number of genotypes varying in resistance to Al have rarely been done (e.g. Ryan et al., 1995b). With eight maize genotypes showing a significant differential resistance to Al and using an Al concentration (40 μM) that best discriminated the genotypes on their sensitivity to Al stress (Chapter 2) we found an exponential-like relationship between citrate exudation rate and root elongation inhibition. Higher citrate exudation into solution seemed to be accompanied by less inhibited root elongation. If maize plants are using root exudation of citrate as a mean of protecting their roots from negative effects of Al, then genotypes with greater ability to exude citrate will be more resistant to the effects of Al. This is confirmed with our eight Brazilian maize cultivars as tested.

A sustained exudation of citrate into the small volume of the root apoplast and rhizosphere will lead to increased local concentrations of this organic anion. Once citrate has left the root cells, it will rapidly form complexes with Al, successively in the apoplast and rhizosphere, reducing Al^{3+} activity and toxicity at local target sites such as cell walls and cell membranes (Suhayda and Haug, 1986). Furthermore, accumulation of citrate outside root cells may create a barrier to the rapid penetration of Al into the sym-

plast. A high local concentration of citrate in conjunction with its relatively high capacity to chelate Al suggests that citrate may account for a mechanism of Al exclusion and detoxification at the root cell surface. The fact that a specific transporter does not exist for the uptake of neutral Al-complexes from solution into maize root cells (Jones and Darrah, 1995) also supports that citrate is involved in an exclusion mechanism of Al^{3+} at the root cell surface.

The spatial localisation of citrate exudation, assessed in intact roots, demonstrated that Al-induced exudation of citrate occurred distinctly along the main root axis of maize seedlings. Measurements of high exudation rates were largely dependent on the presence of root apices while only small amounts of citrate were measured in more mature zones of maize roots without branches. It is clear therefore that the largest part of the plant investment in root exudation of organic anions is being done in the regions of root meristems, where apparently Al accumulates in higher concentrations (Piñeros et al., 2002; Rincón and Gonzales, 1992). This finding also suggests that only cells in a certain stage of development are able to release great amounts of citrate in response to Al stimulus. This pattern of citrate exudation is confirmed by other studies showing that exudation of organic anions induced by Al toxicity but also by P deficiency is mostly localised within the root apex (Delhaize et al., 1993; Hoffland et al., 1989; Pellet et al., 1995). Although these studies have not assessed the whole length of the root, they did show a great predominance of the root apex over the more mature root zone behind the apex.

The results of the present study contrast with those of a recently published study by Piñeros et al. (2002). These authors showed, also with maize roots, fairly constant high Al-induced citrate exudation rates up to a distance of 60 mm from the root apex. Although they claimed that differences between their study and previous study with maize (Pellet et al., 1995) might have been due to genotypic variation among the maize lines studied, it seems that differences in the methodologies employed are more likely to explain these different results, now including ours. The methodology Piñeros et al. (2002) used may have had major implications on the results obtained and a further discussion on that is worthwhile. They studied the spatial localisation of citrate exudation with root segments that were excised and pre-treated for 4 h prior to a 6-h exudation period. It is questionable whether exudation of citrate by excised root segments corresponds with the actual situation once longitudinal transport within the root is no longer possible. Interruption in the longitudinal transport will have direct implications in the transport of assimilates and in the supply of energy to the sites of citrate exudation. It is also clear that by sectioning the root two artificial ‘open ends’ are created in each root segment. Through these open surfaces Al can possibly easily penetrate into the root tissues and stimulate citrate synthesis and release. As shown by the authors in the same paper, cells of the root cortex as well as those of the root stele respond to Al stimulus by releasing

citrate. The high Al-induced citrate exudation by older root zones (e.g. 60 mm from the root tip) is also questionable considering that Al penetrates much less into those root parts, results that were also shown by the authors in the same paper. The use of intact roots in the current study assured that the longitudinal transport of citrate in the root and the supply of assimilates from other parts of the plant (roots) to the sites of citrate exudation were maintained during the course of the experiment. The results presented here seem therefore to describe a more realistic root system.

Protection of the root by citrate seems more conceivable in the places of high exudation rates. In the root zones behind the root apex, either citrate exudation rate is high enough to protect this less Al sensitive region or the root that is now at the position occupied by the root apex in the near past will take advantage of the citrate exuded and still left in the soil at that time. Less Al accumulated in this region will correspond with a weaker competition with nutrients, with direct effects on nutrient uptake along the root axis. For example, Burley et al. (1970) showed that basal parts of seminal roots of maize absorb phosphate and translocate it to the shoot very effectively. Furthermore, lateral roots and basal zones of maize roots are the dominant zones of uptake and translocation of nitrate in maize seedlings (Lazof et al., 1992).

In the root region containing lateral roots, the dynamics of a root growing and releasing citrate is believed to be different. Citrate released in this zone will create a cylindrical microenvironment around the root cylinder and contribute to the protection of the root system as a whole in at least two ways. Firstly, citrate released by the root apices of the lateral roots will protect their own tissues, thus conferring them conditions for a less impaired growth and normal functioning in nutrient and water uptake. Secondly, citrate released from young and short laterals roots will, temporarily, directly protect the main root as well. This protection will be of remarkable importance during the lateral root emergence, a period of lower resistance to penetration of Al via the apoplast into the stele (Rasmussen, 1968). As laterals will be growing, citrate exuded by their apices will no longer directly protect the main root axis, because the cylindrical environment around the main root where citrate is exuded is now at a greater distance than it was with short laterals. Probably this mantle with citrate will create a barrier for monomeric Al to move as such from the bulk soil to the outer surface of the main root. In the mantle monomeric Al will be converted into Al-citrate and subsequently be further transported to the root in this non-toxic form.

In conclusion, aspects of the Al-induced exudation of citrate taken together with the good correlation between citrate exudation and inhibition of root growth indicate a potential role of citrate in a mechanism of Al resistance in maize. A more quantitative evaluation of the findings of this work will be presented in a succeeding chapter. Special attention will be paid to the potential of citrate exudation rates, as measured in the pres-

ent work, to complex Al at sensitive target sites under local conditions. This must indicate the effectiveness and possible restrictions of root citrate exudation as a resistance mechanism to Al with maize.

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Chapter 4

The effect of external aluminium on the root citrate status of an aluminium resistant maize genotype[♣]

Abstract – Despite intensive research on the exudation of citrate by roots as a possible mechanism underlying Al resistance of certain species or cultivars, little attention has been paid to the effects of excess Al on the dynamics of processes as internal root citrate synthesis, translocation, and accumulation. Furthermore, a relationship between internal root citrate concentration and rate of citrate exudation remains speculative. In the present work, we sought the literature about the effects of Al on processes involved in the status of citrate in plant roots, specifically biosynthesis, transport, accumulation, and exudation. In addition, we tested experimentally some of the theories described in literature by studying the effects of external Al on the internal root citrate status and associated root citrate exudation rates along intact root axes of an Al resistant maize genotype. External Al led to higher internal root citrate concentrations. Simultaneously, Al increased the citrate permeability of root cells, but this change in permeability seemed restricted to the regions of the root presenting apices. Consequently, root apices of the Al resistant maize genotype showed enhanced rates of citrate exudation under conditions of Al stress. This exudation followed a particular pattern along the root axis with a well-defined longitudinal distribution, and when compared to the corresponding distribution of internal citrate concentration, indicated that the concentration of citrate itself is not the driving force for citrate exudation from roots. It is suggested that dynamics of processes involved in citrate production, transport, and exudation operate differently along the longitudinal axis of the root and the pattern of citrate accumulation within Al-treated roots observed at the end of the Al treatment period reflected the net result of a combination of these processes.

Key words: aluminium resistance, aluminium toxicity, citrate synthesis, exudation, organic anions, *Zea mays*

[♣] with Willem G. Keltjens

Literature Review – Introduction

Organic anions of low molecular weight have been often mentioned as playing a fundamental role in certain mechanisms evolved by plants to cope with environmental stresses like aluminium (Al) toxicity and phosphorus (P) deficiency. For example, several plant species are able to release organic anions from their roots in response to toxic Al species present in the rooting medium. Because organic anions can carry varying negative charge, they can form complexes with Al thereby reducing its activity in solution and toxicity to roots (Jones, 1998). Some of these organic anions are involved in energy production as intermediates in the tricarboxylic acid (TCA) cycle (e.g. citrate, malate), while others present in cells (e.g. malate, malonate, oxalate) are directly or indirectly involved in many other metabolic processes including the assimilation of carbon and nitrogen, the regulation of cytosolic pH and osmotic potential, the maintenance of electric neutrality during excess nutrient cation uptake, and the supply of energy to symbiotic bacteria (Lambers et al., 1998; Marschner, 1995; Ryan et al., 2001).

Among the organic anions cited above, citrate is one often present in root exudates of plants suffering either from P deficiency or Al toxicity. Citrate appears to be the primary organic anion released by roots of maize plants grown under Al stress conditions (Jorge and Arruda, 1997; Pellet et al., 1995), representing as much as about 70% of the total organic anions exuded under such conditions (Chapter 3). This specific release of citrate in response to excess external Al has been shown to be accompanied by rises in the internal root concentration of citrate (Gaume et al., 2001; Pellet et al., 1995). After 48 h of exposure to 6 μM Al^{3+} activity, roots of maize seedlings had a citrate concentration 50% higher than that observed in roots grown in control solutions (Pellet et al., 1995). Although the exudation of citrate by roots exposed to Al has been well characterised, the corresponding Al-induced changes in citrate production and in the citrate status of roots have not yet been comprehensively studied (Ryan et al., 2001). A diagrammatic representation of carbon pathways relevant to the status of citrate in root cells is presented in Figure 1. Since citrate is an intermediate metabolite in the TCA cycle, its accumulation in root cells may be the result of an increase in its synthesis and/or of a reduction in its conversion into isocitrate, the succeeding metabolite in the TCA cycle. Thus, enzymes directly controlling the status of citrate in root cells are citrate synthase (CS) and aconitase (Aco) through synthesis and consumption of citrate respectively, and any effect of Al on the activity of either of the two enzymes would interfere directly in citrate metabolism. Besides these two enzymes, changes in the activity of the enzyme phospho*enol*pyruvate carboxylase (PEPC) could also have a marked effect on carbon supply and thus on citrate metabolism (Ryan et al., 2001). In the scarce literature dealing with pos-

sible effects of excess external Al on relevant enzymes (Li et al., 2000; Ryan et al., 1995a) there is no direct evidence that accumulation of citrate induced by Al is caused by enhanced activities of PEPC or CS, concomitantly or not with reduced activity of Aco or isocitrate dehydrogenase (ICDH). Disturbance of other known biochemical processes induced by Al is also unknown.

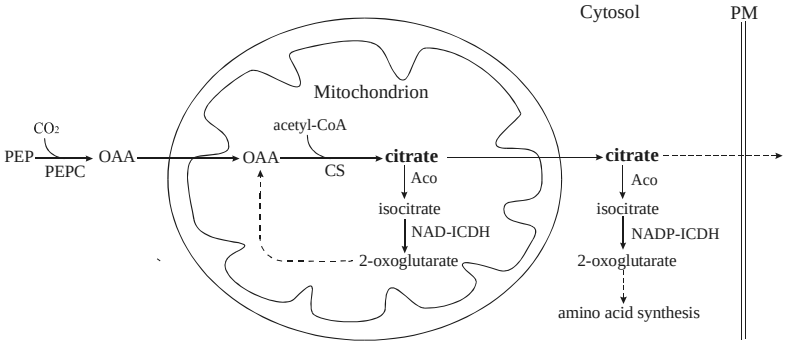

Figure 1. Diagrammatic representation of carbon pathways in plant cells relevant to the citrate status of root cells. Aco, aconitase; CS, citrate synthase; NAD-ICDH, NAD specific isocitrate dehydrogenase; NADP-ICDH, NADP specific isocitrate dehydrogenase; OAA, oxaloacetate; PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase; PM, plasma membrane (after Takita et al., 1999).

Comparatively, aspects of altered organic anion metabolism and their dynamics within the plant induced by P deficiency have been more intensively studied lately (Ryan et al., 2001). These studies have revealed a multitude of changes induced by P deficiency that ultimately results in accumulation of organic anions in the root, and eventually in their exudation to the external medium. For instance, organic anion accumulation in roots and shoots of oilseed rape and in the cluster roots of white lupin coincided with enhanced activities of PEPC, CS, and malate dehydrogenase (MDH), the latter an important enzyme for malate synthesis (Hoffland et al., 1992; Johnson et al., 1994, 1996a,b). Neumann and Römheld (1999) also found a 30% decrease in Aco activity in roots of P deficient plants of tomato, chickpea, and white lupin, which suggests that citrate accumulation may depend on the regulation of both synthetic and catabolic reactions, as explained above. Furthermore, the organic anions accumulated in the roots may be partly originated from the shoots (Hoffland et al., 1992). With plants of oilseed rape

suffering from P deficiency, these authors demonstrated that citrate produced in the shoot was transported via the phloem towards the roots where it was accumulated in the exudation region. Contrary to citrate, no increase in the concentration of malate was observed in the phloem sap of plants suffering from P stress, leading the authors to suggest that malate is probably newly synthesised in the cells of the accumulating root segment from sugars imported from the shoots.

It is now well established that monomeric Al species trigger the release of organic anions from roots with a number of plant species (Ma, 2000; Ryan et al., 2001). The mechanisms mediating the transport of organic anions from the cytosol, where they can accumulate to high concentrations, to the outside medium have been investigated. Initial studies with anion channel antagonists (e.g. niflumic acid, ethacrynic acid), suggested the involvement of anion channels in the transport of malate through the plasmalemma of apical root cells of wheat (Ryan et al., 1995a). Physiological studies have recently revealed a role for ion channels in the exudation of organic anions also in maize roots (Kollmeier et al., 2001). According to Ma (2000), the initial induction of organic anion release occurs in two distinct ways, named pattern I and II. In pattern I, there is no discernible delay between the moment of addition of Al and the onset of the release of organic anions. Activation of an anion channel located on the plasma membrane by Al is a possible mechanism responsible for this rapid release (Delhaize and Ryan, 1995). In pattern II, there is a marked lag phase between the addition of Al and the onset of organic anion release and the action of genes related to the metabolism and exudation of organic anions seems to be involved in this pattern.

Attempts to link internal root concentration of organic anions with root exudation rates have been made, with an implicit assumption that higher internal concentrations would lead to enhanced exudation. However, in many species where organic anion release is stimulated by Al, no correlation is apparent between internal concentration and rate of exudation (Delhaize et al., 1993; Pellet et al., 1995). However, considering that both features, internal concentration and exudation rate, vary significantly along the length of a root (Chapter 3; Jones and Darrah, 1995), indeed much caution must be shown when comparing organic anion concentration and exudation rates of whole root systems.

In the present study we (*i*) sought the literature about the effects of Al on processes involved in the dynamics of citrate in plant roots, specifically biosynthesis, transport, exudation, and accumulation; and (*ii*) tested experimentally some of the theories described under (*i*). We therefore investigated the effects of Al on the internal root citrate status and associated root citrate exudation rates along intact root axes of an Al resistant maize genotype. Theory and experimental findings will be linked and finally be discussed.

Materials and methods

Plant material and growth conditions

The maize genotype CMS36 that has shown a relative superior resistance to Al (Chapter 2) and relative high rates of Al-induced root exudation of citrate (Chapter 3) was used in this study. Roots of seedlings of this genotype were grown axenically to preserve organic compounds exuded by roots and their potential role in protecting the roots against the harmful effects of Al. Thus, all materials used in this experiment were autoclaved and successive treatments were carried out in a laminar-flow hood. To sterilise the surface of the seeds, they were immersed for 1 min in 96% ethanol, soaked for 1 h in a solution containing 1.5% sodium hypochlorite (from commercial bleach) + 1% Tween 20, and subsequently incubated for 15 min in a 1.5% sodium hypochlorite solution. After each treatment the seeds were rinsed three times with sterile demineralised water. To check for eventual microbial contamination surface-sterilised seeds were germinated on nutrient agar plates prepared with a 1 m*M* $CaSO_4$ solution. The plates were placed in a dark chamber at 25 °C for 90 h.

After germination, uncontaminated seedlings were individually transferred to Petri dishes (∅ 94 mm) containing 50 mL of a sterile basal nutrient solution with pH 4.0 and the following chemical composition (m*M*): 1.0 NH_4NO_3, 0.005 NaH_2PO_4, 0.5 K_2SO_4, 0.5 $CaCl_2$, 0.125 $MgSO_4$, and (μM): 46 B, 0.3 Cu, 286 Fe (as Fe-EDTA), 0.1 Mo, 9.2 Mn, 0.8 Zn. The nutrient solution was adjusted to pH 4.0 with 0.1 *M* HCl and autoclaved before the addition of filter-sterilised Fe-EDTA stock solution. In each Petri dish, the roots of one seedling were grown horizontally oriented whereas the shoot was grown vertically through a small notch made in the edge of the lid. The notch was sealed with lanolin and the Petri dish wrapped with foil and transferred to a growth chamber where the seedlings were grown for 3 days at 20 °C under a regime of 16 h light (light intensity 80 W m^{-2})/8 h dark.

Aluminium treatments and collection of root exudates

Aluminium was added to the sterile nutrient solution without Fe-EDTA to reach concentrations of 0 and 40 μM Al. Al was diluted from a filter-sterilised stock solution containing 10 m*M* $AlCl_3$ and 0.1 m*M* HCl. The Al treatment solutions (50 mL) were applied to the seedlings in the same Petri dishes in which they had been growing during the preceding 3 days, replacing the old nutrient solutions. Seedlings were grown for 24 h in the Al treatment solutions before the collection of root exudates started.

The divided-root-chamber technique, described by Ryan et al. (1993) and Delhaize et al. (1993), was used to study the exudation of citrate along the longitudinal axis of the

entire main seminal root of the maize seedlings. After 24 h of Al pre-treatment, seedlings were transferred to large Petri dishes (∅ 145 mm) where they had their roots spatially divided using plastic rings (∅ 13 mm). These plastic rings were placed all over the main seminal root of each seedling covering it from the root apex to the most basal section of the root. A thin layer of vaseline was used to seal the space between the plastic ring and the bottom of the Petri dish and a layer of agar was poured around each plastic ring to hold it over the root. Each plastic ring isolated either an apical 10-mm or a 13-mm long root segment from the rest of the root system forming an individual chamber. An aliquot of 0.5 mL of Al treatment solution was applied to each chamber and with 50 mL of the same treatment solution the rest of the root system was covered. The Petri dish holding the system was closed with a lid to prevent contamination and evaporation of the treatment solutions, wrapped with foil, and transferred to the growth chamber. The solutions enclosed by the rings (diluted root exudates) were collected after 4 h, and stored at −18 °C until analysis. An 8-μL aliquot of the diluted root exudate was plated on nutrient agar and incubated in a dark chamber at 25 °C for 7 days to check for eventual microbial contamination. Contaminated root exudates were discarded. The number of lateral roots enclosed by each plastic ring on the more basal section of the root was counted under a binocular (3.2 × magnification) and the root segments enclosed by the different rings were saved separately for analysis of citrate concentration in the tissue of the different root segments.

Two or three replicate seedlings per treatment were prepared per experiment and the entire experiment was repeated once. Because the seedlings used did not have roots of the same length, the number of plastic rings used to cover the entire main seminal root varied among replicate seedlings. Hence, the exudation values measured in the plastic rings could not be directly averaged across replicates neither compared between treatments, unless the number of rings used per root was the same. To overcome this problem, we considered each plastic ring as representing a percentage of the total length of the root. Therefore, the total root length was divided in 100 units (= 100%) and the citrate exudation value of each ring was repeatedly attributed to every unit that such ring represented. Exemplifying, for a seedling in which nine rings were used to cover the whole root, each ring represented eleven units (= 11%) and the total of nine rings completed 100 units. Once every root had the same relative length, the average of every unit (1%) was calculated among the replicates of the treatments. This procedure was also used for the data on internal concentration of citrate of the root segments.

Extraction of citrate from roots and citrate analysis

After collection of the diluted root exudates, the root segments enclosed by the rings were excised, blotted gently, weighed, and extracted individually according to Delhaize

et al. (1993). Briefly, root segments were extracted with a mortar and pestle in 1 mL of ice-cold 0.6 N perchloric acid. The extract was centrifuged at 15,000 × *g* for 5 min, and 0.9 mL of supernatant solution was collected and neutralised with 80 μL of K_2CO_3 (690 g L^{-1}). The neutralised solution was centrifuged at 15,000 × *g* for 5 min, and an aliquot of the supernatant solution was assayed for citrate.

In the diluted root exudates as well as in the extracts of root tissues, citrate was assayed by using enzymatic methods. A sample of 0.66 mL was incubated with 0.33 mL of buffer (Glycylglycine, 0.51 mol L^{-1}; Zn^{2+}, 0.6 mmol L^{-1}; pH 7.8), 8 μL of NADH (6 mmol L^{-1}), and 7 μL of a mixture of malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) (0.5 mg MDH mL^{-1}; 2.5 mg LDH mL^{-1}). Oxidation of NADH, triggered by the addition of 7 μL of citrate lyase (40 U mL^{-1}), was monitored by UV spectroscopy at 340 nm on a chart recorder and is directly proportional to the amount of citrate in the sample (Boehringer Mannheim, 1984).

Results

Characterisation of the root material used

Maize seedlings had one long seminal root derived from the radicle and typically three to five shorter seminal roots at the time the plastic rings were installed. Only the main (longest) seminal root of each seedling was studied. It was a fully developed root about 120 mm in length and presenting short (< 5–6 mm) lateral roots in the more mature part of the root. The latter part of the root represented about 30% of the total length. The remaining of the root consisted of the root tip and of an intermediate zone, the part between the root tip and the zone with laterals. The apical zone of the root, enclosed by one plastic ring, represented about 15% and the intermediate zone about 55% of the total root length.

Al effects on internal root citrate concentration

Citrate was unequally distributed along the length of the main seminal root growing in control solution (0 μM Al). A remarkable accumulation of citrate was observed in the apical region of the root (about 700 nmol g^{-1} root fresh weight), contrasting with much lower concentrations in the more mature regions, especially in that just behind the apical region (about 150 nmol g^{-1} root fresh weight; Figure 2A). Seedlings exposed to 40 μM Al showed a rather homogeneous spatial internal distribution of citrate along their main root, without any part of the root axis showing significant higher concentrations (Figure 2C). Apart from being more homogeneous, the concentration of citrate in roots at 40 μM Al was higher than in roots at 0 μM Al. Compared to the average concentration of citrate

in the distinct root segments of a single root at 0 μM Al, the roots at 40 μM Al had an average concentration of citrate about 40% higher.

The total citrate contents per segment and per root were calculated based on the fresh weight of the root segments, and the summarised results are presented in Table 1. Because the root axes were homogeneous in radius with root segments of similar fresh weight, the content of citrate in the segments along the root axis followed a similar trend to the internal concentration. The citrate content of the apical 10 mm of the root accounted for about 15–20% of the total citrate content of roots (Table 1), resembling the 15% of the total root length that the root apex represented.

Al effects on citrate exudation

Maize roots released only small amounts of citrate into the external medium when growing in the absence of Al. However, exposure to 40 μM Al greatly stimulated the exudation of citrate from roots (Table 1; Figure 2B,D). Roots grown at 40 μM Al exuded 7 times as much citrate as that exuded by roots at 0 μM Al during the 4-h period of root exudate collection. This difference between the two Al treatments would be even bigger (10 times as much) if only the release at the apical 10 mm root segment was considered. Exudation of citrate in response to Al followed a particular pattern along the root axis, with a well-defined longitudinal distribution. The highest exudation rates were observed in the apical region, a sharp decrease in the direction of basal zones, and a rise again in the zone presenting lateral roots. The sites of highest exudation were therefore closely associated with the presence of root apices, either belonging to the main root or to the lateral roots. Contrary to the distribution of the internal citrate content, where the apical 10 mm of the root accounted for 15–20% of the total root citrate, this apical part accounted for even 44% and 62% of the total citrate exuded by the entire root in 4 h (Table 1).

Discussion

Considerable variation exists in internal concentration of organic solutes, including organic anions, within a single plant root (Jones, 1998). The remarkable accumulation of citrate in the root zones presenting apices, especially in the main root apex, observed in our study with control maize seedlings may conceivably be the result of higher enzyme activity in these parts of the root, similarly to that observed in roots of rye (Li et al., 2002). These authors reported that the activity of CS in roots of rye was 110% higher in the apical 5 mm of the root than in the subsequent 5–20 mm behind the tip. Furthermore, the high demand for photosynthates in the growing parts of the root due to the formation

of new root biomass may also explain the accumulation of organic solutes within the apical part of both seminal and lateral roots, accompanied by high rates of metabolic activity and a concomitant consumption of O_2 (Jones and Darrah, 1996).

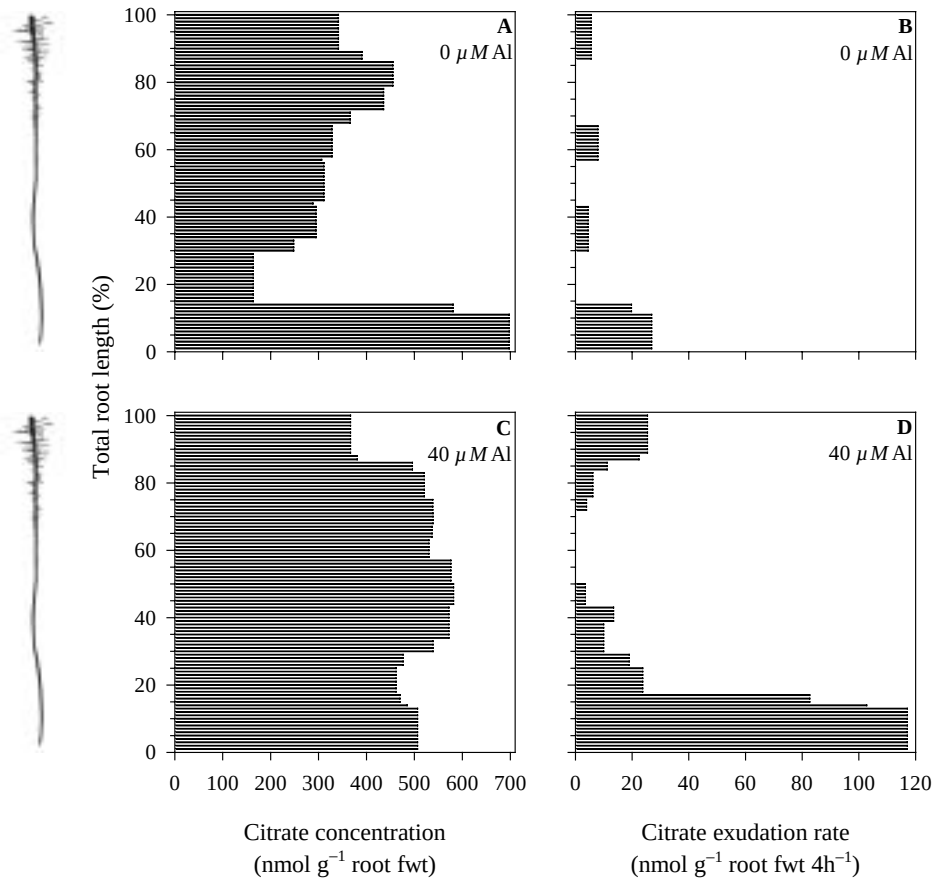

Figure 2. Distribution of citrate concentration (A, C) and citrate exudation (B, D) along the main root axis of the Al resistant maize genotype CMS36 exposed to 0 or 40 μM Al. On the Y-axis 100 values are plotted. They represent the average internal citrate concentration and citrate exudation rates along the root axis of 5 replicates calculated as explained in Materials and methods. Root axes as used in the experiments are schematised on the left-hand side for reference.

Table 1. Citrate status and citrate exudation rate by roots of the Al resistant maize genotype CMS36 grown at two concentrations of Al (0 and 40 μM) for 28 h. The values of citrate content as well as those of citrate exudation corresponding to the root segments enclosed by each plastic ring were summed to calculate the total values per individual root

Root zone	Citrate content (nmol)		Citrate exudation (nmol $4h^{-1}$)	
	Al 0	Al 40	Al 0	Al 40
Whole root	21.2 ± 3.17	31.9 ± 2.80	0.27 ± 0.09	1.96 ± 0.27
Apical 10 mm segment:				
absolute	4.04 ± 0.52	5.16 ± 0.58	0.12 ± 0.08	1.21 ± 0.18
relative (% of whole root)	19	16	44	62

The strong citrate accumulation in roots of maize seedlings treated with Al confirms results from our previous study where plants of the maize genotype CMS36 submitted to 100 μM Al had a root concentration of citrate and malate that was twice as high as that of plants growing in control solutions (Chapter 2). These significant changes in the root citrate status might be seen as a consequence of an Al-induced metabolic disorder, where changes in synthesis and/or consumption of citrate led to an increased accumulation of this metabolite in the root cells. Few studies have tried to elucidate the biochemical pathways that are being disturbed by Al. Attempts to link the accumulation and exudation of root citrate and malate observed under conditions of Al stress with alteration in the activity of enzymes relevant to citrate and malate metabolism have not been successful and indicated that the pattern of alteration may differ between species (Li et al., 2000; Ryan et al., 1995a). Because citrate is a simple intermediate metabolite in the TCA cycle and the enzymatic machinery for its production is already present in the cell, it is likely that cells along the whole root responded to Al through an altered production and storage of citrate in times of Al stress, independently of their differential ability to release part of this citrate to the external medium (Figure 2). Assuming that the aerial parts of the plant would also have their organic anion metabolism altered by Al, and that this response would occur within the initial hours of exposure to Al, it is also possible that part of the citrate present in roots might have been originated from the shoots, like demonstrated with plants of oilseed rape suffering from P deficiency (Hoffland et al., 1992). Thus, a basipetal transport of citrate synthesised in the shoots towards the exudation parts of the root would contribute to the increased amounts of citrate in these root tissues. However,

a downward transport of citrate produced in the shoots towards the roots will probably not contribute to a root citrate accumulation, neither a root citrate exudation immediately after Al addition. This because Al transport from root to shoot will take some time. Both uptake of Al by the root and subsequent transport to the shoots are minimal and also slow. Consequently, Al cannot be expected to be the trigger to initiate increased citrate synthesis in the shoot part of a plant soon after Al exposure.

Besides increased accumulation, a stimulated release of citrate was also observed at 40 μM Al, which resulted in a partial loss of root citrate to the outer medium. The citrate lost by the entire root axis in 4 h represented about 5% of the total citrate content of the root. This fraction could represent much more of the citrate content (as much as about 25%) if only the apical root segment was considered. A sustained synthesis of citrate is therefore required to sustain such a high rate of citrate exudation, which would otherwise completely deplete the citrate reserve of that root segment within a few hours. Apart from synthesis, there might be other processes that are contributing to a continuing exudation of organic anions, like for example an Al-induced transport of organic anions from other root segments to root tips. For that, a regulation mechanism controlling synthesis, accumulation, and exudation of citrate to the external medium is expected.

The pattern of citrate concentration within Al-treated roots observed at the end of the 28-h Al treatment period reflected the net result of a combination of these different processes [i.e. cell metabolism (biosynthesis and decomposition), reallocation and storage, and exudation] responsible for the dynamics of citrate within the root. In this study we could not ascertain the contribution of each process to the overall citrate status of the root but a simple illustration of the differential contribution of the root segments and the different processes in the dynamics of citrate could be done by simulating that no citrate would be released to the external medium, being kept inside the respective root segment instead, and finding a similar pattern of accumulation of citrate in the main apical region of the root for the two Al treatments (Figure 3).

The results presented here strongly indicate that the internal concentration of citrate itself is not the driving force for citrate exudation from roots. Evidence for that is firstly the fact that the internal concentration of citrate was even higher in the main root apex of control plants than in the root apex of plants exposed to Al, while only a baseline exudation was observed from roots of control plants; secondly, although an overall increase in citrate concentration was observed along the whole root axis after addition of Al, the citrate exudation was largely confined to specific sites of the root axis after Al exposure. Interestingly, the root parts with highest rates of Al-induced exudation were those where citrate was accumulated at highest when Al was absent. It becomes very likely therefore that Al induces a high permeability to citrate in root cells (Jones, 1998). This induction of exudation seems highly specific to Al, once other trivalent metals like lanthanum

(La^{3+}), gallium (Ga^{3+}), indium (In^{3+}), and ytterbium (Yb^{3+}), were not able to induce exudation of organic anions from roots of several plant species (Li et al., 2002; Ma et al., 1997; Ryan et al., 1995a). But the fact that exudation of citrate as induced by Al was most confined to the regions of root meristems indicates that only cells in a certain stage of development are able to release citrate in response to such stimulus. Anion channels that are specifically activated by extracellular Al^{3+} have recently been reported in protoplasts isolated from root apices of Al resistant wheat (Ryan et al., 1997; Zhang et al., 2001), and maize (Kollmeier et al., 2001; Piñeros and Kochian, 2001). Because higher activity of anion channels have been found in an Al resistant than in an Al sensitive maize genotype, coinciding with higher exudation in the first, these transport systems have been speculated as mediating the Al activated root organic anion release in maize (Kollmeier et al., 2001), even though direct permeability of these channels to organic anions have not been experimentally proved.

It is interesting to note that although high concentrations of citrate are found in the root tip before the addition of Al, rapid release of great amounts of citrate soon after exposure to Al is not verified in maize (Chapter 3; Pellet et al., 1995). The exudation starts slowly and a lag phase of up to 24 h is observed between the moment of Al addition and that of maximal rate of citrate release. It is suggested that a slower Al-induced response may involve induction of genes implicated in organic anion metabolism (biosynthesis and decomposition), anion channel on plasma membrane and/or tonoplast, or transport of organic anions from mitochondria (Ma et al., 2000; Piñeros et al., 2002), contrary to the rapid activation (i.e. opening) of anion channels in exudation Pattern I. The high internal root citrate concentrations induced by Al lead us to speculate that in addition to the Al exclusion mechanism based on the Al activated exudation of citrate and external complexation of Al, an internal Al detoxification (Al tolerance) mechanism may operate in maize, constituting a second mechanism of Al resistance in this plant species.

Researchers have manipulated the biosynthetic capacity of cells expecting they will produce and accumulate higher amounts of organic anions, with a hope that this will ultimately result in an altered exudation profile of a given genotype. The most well-known example of an achievement in this direction is the successful work of de la Fuente et al. (1997), who transferred a citrate synthase gene from the bacterium *Pseudomonas aeruginosa* into tobacco and papaya and recorded an increased accumulation of citrate, accompanied by increased citrate efflux into the rhizosphere and a somewhat increased resistance of transformed plants to Al. On the other hand, Delhaize et al. (2001) argued that expression of the *P. aeruginosa* citrate synthase gene in plants is unlikely to be a robust strategy for enhancing the Al resistance of plants. Delhaize and coworkers generated tobacco lines that expressed the citrate synthase gene from *P. aeruginosa* and an activity of CS up to a 100-fold greater level than the non-transformed plants

but without showing increased accumulation of citrate in roots or increased Al-induced exudation from roots.

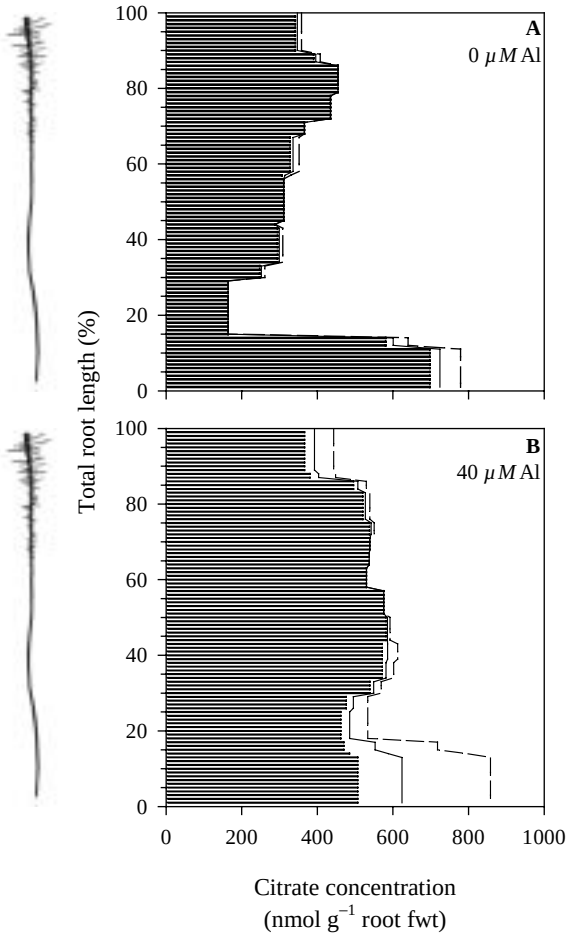

Figure 3. Distribution of citrate concentration along the main root axis of the Al resistant maize genotype CMS36 exposed to 0 or 40 μM Al for 28 h (bars) and simulated root concentration considering that the citrate exuded for 4 h (continued line) or 12 h (dashed line) would be kept inside the root segments they were exuded from. Root axes as used in the experiments are schematised on the left-hand side for reference.

As pointed out by Rengel (2002), increase of synthesis and, eventually, accumulation of organic anions in root cells is not a guarantee for high rates of root exudation. Rather, effective exudation of organic anions into the rhizosphere relies on at least three processes: signalling sequence, effective biosynthetic machinery producing relatively large amounts of organic anions, and a membrane transporter that allows transfer of organic anions into the root apoplast and rhizosphere.

We conclude that with the Al resistant maize genotype as tested, external Al leads to enhanced internal root citrate concentrations. Simultaneously, Al seems to increase the citrate permeability of root cells, but this change in permeability seems to be restricted to the apical part of the root. Consequently, under conditions of Al stress root apices of the Al resistant maize genotype showed enhanced rates of citrate exudation.

Chapter 5

Long-term effects of aluminium exposure on nutrient uptake by maize genotypes differing in aluminium resistance[♣]

Abstract – Genotypic differences in resistance to aluminium (Al) found in many plant species grown in conditions of Al stress seem to include differences in Al-induced inhibition of absorption and utilisation of nutrients. Aiming to study the effects of Al on nutrient uptake of maize genotypes differing in Al resistance and to check whether differences in mineral nutrition under Al stress correspond with differences in resistance to Al in maize, an experiment involving ten maize genotypes differing in Al resistance and two concentrations of Al (0 and 100 μM Al) was established. Total plant (shoot + root) uptake of P, K, Ca, Mg, Cu, Fe, Mn, and Zn was determined in maize plants after 14 days of growth in culture solution at the two concentrations of Al. The relative uptake [(uptake at 100 μM Al/uptake at 0 μM Al)×100] of the nutrients studied varied from 22% to 157%, indicating the existence of intraspecific variation for such feature in the presence of Al. Generally, Al had negative effects on the uptake of macro and micronutrients. Al effects were most pronounced on the uptake of Ca and Mg with respective reductions of 61% and 72%, when averaged across the ten genotypes. Among the micronutrients the most pronounced effects of Al were noted on Mn and Zn. Despite showing significant reductions in uptake of Ca and Mg, the maize genotypes showed a rather variable sensitivity to the Al stress imposed, which was related to their general resistance to Al previously assessed using root length as indicator. Under conditions of Al stress, genotypes more resistant to Al maintained a relatively higher absorption of both Ca and Mg than those more sensitive to Al, suggesting that the ability of a genotype to maintain a less disturbed nutrient uptake under Al stress can be an important component in resistance to Al.

Key words: aluminium toxicity, calcium, culture solution, magnesium, nutrient uptake, *Zea mays* cultivars

[♣] with Willem G. Keltjens

Introduction

Negative effects of aluminium (Al) on plant growth occur primarily in the root system, where generally Al inhibits root elongation and disrupts the uptake of nutrients. In the root apoplast, where the uptake of nutrients starts, Al competes with nutrient cations, such as calcium (Ca) and magnesium (Mg), for binding sites on the root cortical cell walls and on the outer surface of the plasma membrane, decreasing the concentration of these nutrients in the direct vicinity of the uptake sites and inhibiting their absorption into the symplast (Marschner, 1995). Inhibition of phosphorus (P) uptake occurs due to (co)precipitation of P with Al at the root outer surface and Al bound at cell wall material in the root apoplast. Precipitated with or bound to Al, P remains largely inorganic (= not incorporated into organic compounds) and exchangeable, and is not absorbed or used in the plant metabolism (Clarkson, 1967). Negative effects of Al on the uptake of Ca, Mg, and P, among other nutrients, have been demonstrated in plant species like maize (Clark, 1977; Keltjens, 1995), sorghum (Tan and Keltjens, 1990), ryegrass (Rengel and Robinson, 1989a), and wheat (Keltjens and Dijkstra, 1991).

However, nutrient uptake by different genotypes of a certain species is not equally inhibited when exposed to Al. In this respect they can differ considerably when exposed to a given condition of Al stress. Baligar et al. (1993) studied the effects of soil Al on the uptake of nutrients in 40 sorghum genotypes and reported a large intraspecific variation among them. Besides differing in uptake, the sorghum genotypes showed to differ also in other features of nutrient uptake like nutrient influx into roots and subsequent transport to the shoots. Al resistant genotypes showed higher uptake, influx, and transport of nitrogen (N), potassium (K), iron (Fe), zinc (Zn), P, Ca, and Mg than did the Al sensitive genotypes. These authors reported that the potential of certain genotypes to maintain high dry matter yields under conditions of Al stress was partly due to their capability to keep their nutrient uptake at relative high rates, suggesting that the ability to maintain a less disturbed mineral nutrition under Al stress could be an important component in resistance to Al.

Our objective in establishing this study was to determine the effects of the presence of Al in the rooting medium on the nutrient uptake of maize genotypes differing in resistance to Al and to check (*i*) whether they indeed differ in nutrient uptake when exposed to Al and (*ii*) whether variation in nutrient uptake among genotypes, due to Al exposure, corresponds with variation in Al resistance as described before (Chapter 2).

Materials and methods

Ten Brazilian maize genotypes representing a range of resistance to Al were used to study the effects of Al on nutrient uptake. The ten genotypes chosen belong to the collection of the maize breeding program of the National Research Centre of Maize and Sorghum – EMBRAPA, Brazil. They were selected mainly based on screening experiments carried out at EMBRAPA in culture solution containing different concentrations of Al. Their names are: G1 (64×1143; single cross hybrid); G2 (BR201-M; single cross hybrid); G3 (HD91102; double cross hybrid); G4 (13×1143; single cross hybrid); G5 (CMS36; open-pollinated variety); G6 (11×723; single cross hybrid); G7 (20×723; single cross hybrid); G8 (HD9148; double cross hybrid); G9 (BR106; open-pollinated variety); and G10 (20×22; single cross hybrid). Subsequently, they will be referred to as G1, G2, G3, etc.

Plants of the ten maize genotypes were grown in a culture solution at zero or 100 μM Al. The experiment was conducted under greenhouse conditions in pots filled with continuously aerated culture solution with pH 4.0 and chemical composition (mM): 2.0 NH_4NO_3, 0.0375 NaH_2PO_4, 1.0 K_2SO_4, 1.0 $CaCl_2$, 0.25 $MgSO_4$, and (μM): 46 B, 0.3 Cu, 286 Fe (as Fe-EDTA), 0.1 Mo, 9.2 Mn, 0.8 Zn.

Seeds were germinated in moist quartz sand for 7 days. After germination, the roots were washed to remove the sand and a total of 54 uniform seedlings per genotype was selected for the experiment. Seedlings were wrapped loosely with sponge rubber and mounted in holes of plastic covers (discs) placed on 6-L pots. After a pre-growth for 4 days in the above mentioned culture solution the Al treatments were imposed. The culture solution was replaced by a similar culture solution containing 100 μmol Al L^{-1} or without Al, the latter used as a control. The culture solutions were renewed every other day and 14 days after the Al treatment had started the plants were harvested. Plants of each pot were divided into plants used for total root length measurements and shoot and root biomass production, and plants used for analysis of mineral composition of shoots and roots. Root length measurements were carried out on representative sub-samples of fresh root material using a Comair root length scanner (Hawker De Havilland, Melbourne, Australia). Shoot and roots were oven-dried at 70 °C for 72 h and weighed. Subsequently shoots and roots were ground in a stainless steel grinder and digested in a sequential procedure, using HF, HNO_3, and H_2O_2, in a closed system microwave oven (Novozamsky et al., 1996). Digests were analysed on copper (Cu), manganese (Mn), P, K, Ca, Mg, Fe, Zn, and Al by ICP–AES. The total plant nutrient content (or plant nutrient uptake) was calculated as: [(root dry matter × root nutrient concentration) + (shoot dry matter × shoot nutrient concentration)].

The experimental design was a completely randomised one with three replicates. The treatments resulted from a factorial combination of two concentrations of Al (0 and 100 μM Al) and ten maize genotypes. Analysis of variance was performed for all plant characteristics measured. When significant, the genotype × Al interaction was partitioned into single degree of freedom orthogonal contrasts, where Al concentrations were studied in simple effect comparisons within each genotype. To study the Al resistance as expressed by the ratio [+Al/−Al], the data were log-transformed, since [log(+Al/−Al)] = [log(+Al) − log(−Al)]. Using the 'estimate' statement of General Linear Models procedure of SAS (SAS Institute, 1990), these differences were calculated and the values were further compared using the Tukey test. First-order polynomial equations were fitted to data on the relationship between relative root length and relative uptake of Ca or Mg. Statistical analyses were performed using the SAS software (SAS Institute, 1990).

Results and discussion

Growth of the ten maize genotypes studied was adversely affected by the addition of Al to their rooting medium. Generally they showed significant reductions in total root length and in shoot and root biomass when exposed to 100 μM Al for 14 days (Table 1). However, the ten genotypes differed in resistance to Al in solution. Al caused reductions in root length that varied from 40% in G4 up to 74% in G9. Compared to root length, the Al effects on shoot and root biomass were less severe. Reductions averaged 34% in shoot and 25% in root, apart from an increase of 7% in the root biomass of G4. While inhibition of root elongation is considered the primary or initial response of the plant to Al toxicity (Kochian, 1995), restriction of shoot growth is often considered as a secondary response, being the consequence of root damage and its impaired functioning with respect to water and nutrient uptake. Therefore, compared to the root, there is a delay in shoot response to Al. This could partially explain the difference in magnitude of the inhibition in root length and shoot biomass as found in this experiment. A significant statistical differentiation of the genotypes for Al resistance was only achieved when using root length. Confirming earlier results of Furlani and Clark (1981), in experiments under controlled conditions, root length has reflected better the general ability of a plant to cope with toxic Al than shoot and root biomass and therefore it has been preferred as indicator of Al resistance.

Aluminium had negative effects on the uptake of both macro and micronutrients in all ten genotypes studied with a few exceptions for P, Cu, and Fe. The severity of these effects can be exemplified by the significant reductions in the uptake of K, Ca, Mg, Mn, and Zn found in all ten genotypes tested (Tables 2 and 3), in spite of a significant geno-

type × Al interaction with Ca, Mn, and Zn and nearly significant with K and Mg uptake (statistics not shown). With the macronutrients the negative effects of Al were most pronounced on the uptake of Ca and Mg. The uptake of both nutrients by the ten maize genotypes grown with Al averaged 39% and 28% of the control plants, for Ca and Mg respectively. With the micronutrients, the strongest effects of Al were noted on the uptake of Mn and Zn. Grown at 100 μM Al, all ten genotypes absorbed Mn or Zn in amounts significantly lower than their control plants grown in the absence of Al (Table 3). Unlike Mn and Zn, the genotypes showed wider variations in uptake of Cu and Fe. Genotypes 3, 4, and 5 absorbed these two nutrients in amounts non-significantly different from their control plants.

Table 1. Plant characteristics of ten maize genotypes grown at zero (Al 0) or 100 (Al 100) μM Al in culture solution for 14 days. A significant effect of the addition of Al is indicated in the 'Al 100' columns by ** ($p < 0.01$) or * ($p < 0.05$). Values followed by the same letter within a column are not significantly different at 5% of significance in the Tukey test

Genotype	Root Length[1]		Root Biomass[2]		Shoot Biomass[2]	
	Al 0	Al 100	Al 0	Al 100	Al 0	Al 100
G1	300 a	44** ab	2.10 a	77* a	6.58 ab	68** a
G2	183 b	39** ab	1.37 b	70** a	6.59 ab	61** a
G3	238 ab	47** ab	1.82 ab	87 a	7.03 ab	61** a
G4	210 b	60** a	1.86 ab	107 a	6.72 ab	80* a
G5	241 ab	51** a	1.64 ab	84 a	7.36 a	81* a
G6	177 b	46** ab	1.41 b	83 a	5.88 bc	70** a
G7	209 b	40** ab	1.43 b	71** a	5.07 c	57** a
G8	224 ab	52** a	2.18 a	69** a	6.97 ab	60** a
G9	236 ab	26** b	1.33 b	65** a	5.12 c	62** a
G10	166 b	38** ab	1.41 b	73* a	6.22 abc	62** a
Mean		44		79		66

[1] values are given in m (4 plants)$^{-1}$ in the 'Al 0' column and in % of the control (Al 0) in the 'Al 100' column

[2] values are given in g (4 plants)$^{-1}$ in the 'Al 0' column and in % of the control (Al 0) in the 'Al 100' column

Despite showing significant reductions in uptake of most nutrients, the maize genotypes showed a rather variable sensitivity to the Al stress imposed. With the ten maize genotypes the relative uptake of the nutrients studied varied from 22% to 157%. The existence of intraspecific variations in mineral uptake and utilisation in various crop species in the presence and absence of Al is well documented (Foy, 1984) and our findings confirm these variations to exist also in maize. It is also well documented that increasing concentrations of Al in soil or in nutrient solution negatively affect root influx of nutrients and transport to the shoots, consequently decreasing nutrient accumulation by plants (Foy, 1984), which is in agreement with the results found in our study.

Table 2. Plant uptake of P, K, Ca, and Mg by ten maize genotypes grown at zero (Al 0) or 100 (Al 100) μM Al in culture solution for 14 days. A significant effect of the addition of Al is indicated in the 'Al 100' columns by ** ($p < 0.01$) or * ($p < 0.05$). Values followed by the same letter within a column are not significantly different at 5% of significance in the Tukey test

Genotype	**P**[1]		**K**		**Ca**		**Mg**	
	Al 0	Al 100	Al 0	Al 100	Al 0	Al 100	Al 0	Al 100
G1	27.9	87 a	552	61**a	38.5	44**ab	13.1	31**ab
G2	44.6	77**a	541	54**a	39.2	35**abc	12.2	26**ab
G3	27.6	89 a	535	50**a	42.4	37**abc	14.4	29**ab
G4	26.5	104 a	450	74**a	32.3	60**a	12.6	37**a
G5	28.6	85 a	533	76* a	42.9	41**abc	15.6	30**ab
G6	26.0	94 a	404	63**a	35.2	39**abc	12.9	28**ab
G7	25.1	85 a	410	52**a	34.4	33** bc	11.1	27**ab
G8	31.2	83* a	519	55**a	30.8	48**ab	14.3	29**ab
G9	23.9	84* a	389	53**a	38.0	24** c	12.0	22** b
G10	24.1	81* a	491	46**a	37.1	28** bc	14.1	22** b
Mean		87		58		39		28

[1] values are given in mg of nutrient (4 plants)$^{-1}$ in the 'Al 0' columns and in % of the control (Al 0) in the 'Al 100' columns

The uptake of P and K was less affected by 100 μM Al. Precipitation of P by Al and subsequent accumulation on the root outer surface or in the root apoplast without being

absorbed by the root cells may have contributed to increase the relative high values of total P content of roots of plants exposed to Al, consequently maintaining relative high values of 'apparent' P uptake of these plants. In contrast to the divalent cations Ca and Mg, uptake of the monovalent cation K has been shown to be less inhibited by Al and in certain cases the uptake of K at low concentrations of Al may be even higher than in solutions without Al (Rengel and Robinson, 1989a).

Table 3. Plant uptake of Cu, Fe, Mn, and Zn by ten maize genotypes grown at zero (Al 0) or 100 (Al 100) μM Al in culture solution for 14 days. A significant effect of the addition of Al is indicated in the 'Al 100' columns by ** ($p < 0.01$) or * ($p < 0.05$). Values followed by the same letter within a column are not significantly different at 5% of significance in the Tukey test

Genotype	**Cu**[1]		**Fe**		**Mn**		**Zn**	
	Al 0	Al 100	Al 0	Al 100	Al 0	Al 100	Al 0	Al 100
G1	60.3	54** b	2049	50** c	495	45**abc	246	57**ab
G2	61.7	55** b	1599	57** bc	553	40**abc	260	37** bc
G3	59.7	74 ab	1945	93 ab	650	38**abc	216	53**ab
G4	46.0	157* a	1799	112 a	481	56**a	184	65**a
G5	72.0	86 ab	1780	100 a	654	44**abc	307	48**abc
G6	53.0	49** b	1803	50** c	505	38**abc	247	46**abc
G7	53.3	52** b	1638	50** c	445	39**abc	216	42**abc
G8	58.3	59* ab	2096	55** bc	496	46**ab	250	41**abc
G9	38.0	51** b	1583	44** c	502	28** c	185	32** c
G10	40.3	41** b	1819	46** c	559	32** bc	211	32** c
Mean		68		66		41		45

[1] values are given in μg of nutrient (4 plants)$^{-1}$ in the 'Al 0' columns and in % of the control (Al 0) in the 'Al 100' columns

Our results are in agreement with other reports about Al effects on nutrient uptake by maize (Clark, 1977; Poschenrieder et al., 1995) as well as by other gramineae like sorghum (Duncan, 1987; Tan and Keltjens, 1990), rice (Jan, 1991), and ryegrass (Rengel and Robinson, 1989a). Also, the increased absorption of Cu and Fe found in this study with some genotypes is consistent with the results of Clark (1977), who reported an Al

resistant maize genotype with enhanced uptake of Cu, Fe, Mn, and Zn when grown in a 93 μM Al nutrient solution, contrasting with an Al sensitive genotype that showed a reduced uptake of the micronutrients considered.

Calcium and magnesium have been mentioned in literature as key nutrients involved in the Al toxicity syndrome (Marschner, 1995), and genotypes with the ability to maintain a less inhibited uptake of these two nutrients under Al stress are expected to have a higher resistance to Al. Consequently, genotypes more resistant to Al will accumulate higher amounts of these two nutrients in a given period. To check the existence of such relationship with the maize genotypes tested here, regression analysis was run between the relative uptake of Ca or Mg and the relative total root length, used as the best indicator of Al resistance with the ten maize genotypes. First-order polynomial equations were fitted and the results are shown in Figure 1. Significant positive relations were found indicating that increasingly genotypic resistance to Al was accompanied by higher relative values of Ca and Mg uptake. Genotypes more resistant to Al (e.g. G4 and G5) indeed absorbed relatively higher amounts of both nutrients than the less Al resistant genotypes, i.e. G9 and G10, what might have contributed to a better growth of the plants with consequent higher biomass production and nutrient uptake. In other studies evaluating the toxic effects of Al on the mineral nutrition of plant species, genotypes more resistant to Al also have shown a less impaired nutrient uptake than genotypes less resistant to Al (sorghum, Baligar et al., 1993; maize, Clark, 1977; ryegrass, Rengel and Robinson, 1989a).

Uptake of Mg was most severely depressed by Al and it was further investigated by studying the Mg concentration in shoots of the ten maize genotypes (Figure 2). Grown in absence of Al, genotypes had an average shoot Mg concentration of about 80 mmol kg^{-1} dry matter. When grown with 100 μM Al the shoot Mg concentration was reduced significantly in all genotypes to concentrations of about 30 mmol kg^{-1} dry matter, which are well below the 60 mmol kg^{-1} dry matter reported by Reuter et al. (1997) as the critical Mg concentration for maize. Considering the status of the other nutrients in shoots of maize plants growing in 100 μM Al nutrient solution, Ca was found to be present in marginal concentrations while Zn was present in concentrations in the deficiency range, following values suggested by Reuter et al. (1997). Thus, evaluated at the end of the 14-d period of Al exposure, the ten genotypes showed a general status of nutrient deficiency, which was probably initiated at different stages for the ten genotypes, but led all ten genotypes to the same final status.

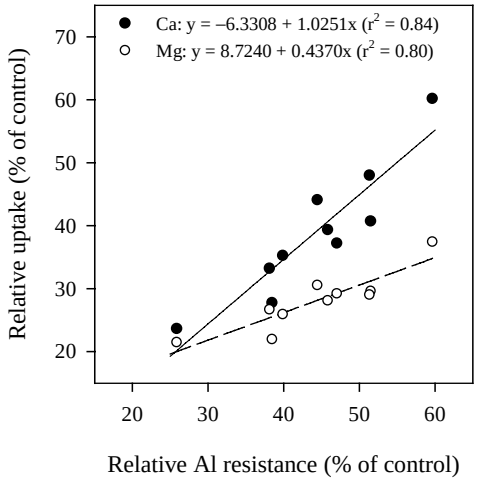

Figure 1. Relationships between relative Al resistance and relative uptake of Ca or Mg of ten maize genotypes grown at zero or 100 μM Al in culture solution for 14 days.

Reduction of nutrient uptake caused by Al as reported here is not only the result of direct or primary effects of Al on nutrient uptake, as explained in the introduction. Firstly, during the 14-d period Al has affected both root length and shoot biomass production of the ten genotypes differently, although significant differences among genotypes were only found in root length (Table 1). Differences in root length will directly affect nutrient uptake by changing plant's uptake capacity, while shoot biomass will be involved by affecting the plant nutrient demand (sink). Clearly, these two effects of Al occurred simultaneously and were collectively taken into account here. Consequently, the higher production of shoot biomass and root length by the resistant genotypes grown under Al stress (Table 1) have certainly contributed for their higher relative values of nutrient uptake (Tables 2 and 3). Secondly, as shown in Figure 2, at the end of the 14-d period, plants of all genotypes showed shoot Mg concentrations far below the critical concentrations for maize. This means that 100 μM Al in solution has induced Mg deficiency and thus also inhibited growth during part of the 14-d period with all ten genotypes. Probably, the moment at which this critical internal Mg concentration was reached was not the same for all ten genotypes. At the moment that Mg deficiency appears, mal-

functioning of the plant will not only inhibit growth but also disturb the process of nutrient uptake.

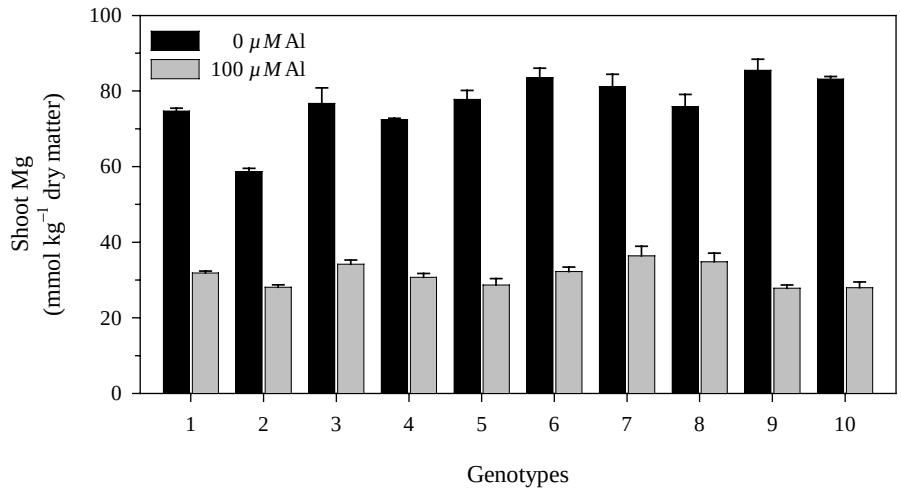

Figure 2. Mg concentration in shoots of plants of ten maize genotypes grown at zero or 100 μM Al in culture solution for 14 days.

Therefore, it was not possible with this study to quantify the specific or direct effects of Al on nutrient uptake, without excluding the reduction in nutrient uptake due to indirect Al effects, i.e. impaired root and shoot growth and Mg deficiency. Therefore, in a next study, we will focus on the short-term effects of Al on nutrient uptake with the same maize genotypes. We will make use of short-term experiments in an attempt to avoid significant changes in root length and root morphology and in internal nutrient status caused by Al. For this purpose genotypes that are clearly different in sensitivity to Al, like G5 and G9, will be selected.

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Chapter 6

Direct effects of aluminium on nutrient uptake by maize genotypes differing in aluminium resistance with special emphasis on the uptake pattern along the seminal root[♣]

Abstract – Aluminium (Al) ions can affect the uptake of plant nutrients in two ways. Indirectly, by reducing root growth (and thus reducing root surface available for nutrient absorption), and directly by competing with nutrient cations for absorption sites on the plasma membrane. To minimise, or even to avoid, the occurrence of these confounding factors, studies on the effects of Al on nutrient uptake should not take long. Besides they should consider plant material whose growth preceding nutrient uptake measurement has not been altered by Al. In the present study short-term (24 + 6 h) effects of exposure to Al on nutrient uptake were investigated along different zones of root axes of maize seedlings. With two maize genotypes exhibiting differential resistance to Al, uptake of calcium (Ca), magnesium (Mg), and potassium (K) was studied by complete seminal roots and along the whole axis of intact seminal roots of seedlings grown with or without Al. Exposure to Al reduced total uptake of Ca and Mg, but not that of K, which was actually stimulated by Al. The negative effects of Al on Ca and Mg uptake were more pronounced in the Al sensitive genotype. The assessment of the spatial localisation of nutrient uptake on the root axis revealed that Al is affecting nutrient uptake widely along the longitudinal axis of the root. The pattern of Al-induced inhibition in nutrient uptake is compared with the pattern of citrate exudation along identical root axes of the Al resistant genotype, assessed in a previous study. Collectively, these results form the basis of a qualitative evaluation of the potential role of organic anions, exuded distinctly along the root axis (Chapter 3), in a mechanism of protection against the adverse effects of Al on nutrient uptake.

Key words: aluminium toxicity, calcium, citrate exudation, magnesium, root axis, *Zea mays* cultivars

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[♣] with Willem G. Keltjens

Introduction

The uptake of nutrients by plant roots starts with the movement of ions and uncharged molecules from the external solution into the cell walls and water-filled intercellular spaces of the root cortex (= root apoplast). While diffusion or mass flow generally drives the movement of nutrients into the root apoplast, their pathways and mechanisms of transport diverge when passing the endodermis and moving into the symplast. Ions bind at specific sites in the plasma membrane (e.g. carriers, transport proteins) and are transported actively into the cytoplasm or cross the plasma membrane passively through ion channels (Marschner, 1995). The rate of ion uptake per unit root length tends to decline as the distance from the apex increases. But this tendency is not unique and varies according to type of nutrient, plant nutritional status, and plant species (Marschner, 1995). Uptake of calcium (Ca) and magnesium (Mg) is higher in apical than in basal root zones, while uptake of potassium (K) and phosphorus (P) occurs more smoothly along the longitudinal axis of the root (Ferguson and Clarkson, 1975; 1976; Kochian, 1995; Marschner, 1995).

While in the root apoplast, cations and anions, present in solution in different concentrations and forms, interact with each other and with electrically charged groups located in the cell walls and on the outer surface of the plasma membrane. These interactions may facilitate or restrict further movement of the ions to the absorption sites of the plasma membrane of individual cells or roots. According to Marschner (1995), binding of nutrient cations to carboxylic groups of the cell wall increases the concentrations of these cations in the root apoplast and thus in the vicinity of the active uptake sites at the plasma membrane, enabling high rates of nutrient uptake. An example of restriction of nutrient uptake is the direct competition between ions for common binding sites. The strength of the interaction between negative binding charges and cations increases with the valency of the ion. Due to preferential binding of polyvalent cations [e.g. aluminium (Al)] they would to a great extent neutralise these negative charges at the cost of other cations with lower affinity (e.g. Ca, Mg) and decrease further accumulation of these cations towards the membrane surface (Ryan and Kochian, 1993).

Aluminium in the rooting medium is able to inhibit the uptake of several nutrients in many plant species (Foy, 1984). Aluminium hydrolyses in solution such that the trivalent Al species (Al^{3+}) dominates in acid conditions (pH < 5). This monomeric cation binds to various inorganic and organic ligands such as PO_4^{3-}, SO_4^{2-}, F^-, organic anions, proteins, and lipids (Kinraide, 1991). With this wide range of interactions, it is not surprising that Al^{3+} can disturb the uptake of nutrients in different ways. While it is generally accepted that binding of Al^{3+} to the lipids and proteins in biological membranes may interfere

with the transport of nutrient into the cytoplasm (Taylor, 1988), Al^{3+} has been shown to compete with nutrient cations (mainly the divalent Ca and Mg) for binding sites in the cell walls and on the plasma membrane, to displace those adsorbed on these sites and to decrease their concentration around the transport sites on the plasma membrane (Rengel and Robinson, 1989b). Besides these competing effects on cations, Al^{3+} probably reduces their absorption also by blocking cation channels (Kochian, 1995). Inhibition of P uptake occurs due to (co)precipitation of P with Al at the root outer surface and Al bound at cell wall material and cell membrane in the root apoplast. Precipitated with or bound to Al, P remains largely inorganic and exchangeable, and is not absorbed or used in the plant metabolism (Clarkson, 1967). However, differences in sensitivity to Al effects on nutrient uptake are observed between genotypes of a certain species, and it was once suggested that increased Al resistance is associated with an increased ability to maintain normal nutrient fluxes and membrane potentials across the plasma membrane of root cells in the presence of Al (Miyasaka et al., 1989). In the work of Huang et al. (1992), addition of Al concentrations (5–20 μM $AlCl_3$) to an Al sensitive wheat cultivar (Scout 66) inhibited Ca^{2+} uptake enormously whereas in an Al resistant cultivar (Atlas 66) Ca^{2+} uptake was relatively unaffected. But genotypic differences in the Al effects on Ca^{2+} uptake were larger in the apical 5 mm of the root than in the region 5–20 mm behind the root tip.

The toxicity of Al^{3+} ions can be reduced through Al complexation with small ligands such as sulphate, fluoride, and organic anions (Kinraide, 1991). Complexation of Al^{3+} by externally applied organic anions (e.g. citrate, malate) has been shown to reduce the impact of toxic Al on crucial cellular components and physiological processes (Keltjens, 1995; Ownby and Popham, 1989; Suhayda and Haug, 1986). Exudation of these compounds from plant roots has been lately implicated in a mechanism of detoxification of Al^{3+} in the root apoplast and/or rhizosphere, and thus in protection of the roots against its harmful effects (for a review see Ryan et al., 2001). But unlike the absorption of nutrients that may occur along the whole extension of the root axis (Marschner, 1995), high rates of Al-induced exudation of organic anions are strictly confined to root zones presenting apices, either the apex of the main root or the apices of lateral roots initiating from the main root (Chapter 3). The protective effect to be conferred by organic anions through complexation and detoxification of Al^{3+} is therefore more likely at places with higher organic anion exudation, probably leaving the rest of the root unprotected against the well-known negative effects of Al ions on nutrient uptake (Keltjens, 1995; Rengel and Robinson, 1989a).

This study was established therefore to investigate the short-term or direct effects of exposure to Al on nutrient uptake along different zones of a root axis. With two maize genotypes exhibiting differential resistance to Al, nutrient uptake was studied (*i*) by

complete seminal roots, and (*ii*) in distinct regions of seminal roots of plants grown with or without Al. The investigation of the spatial distribution of the Al effects along the root axis on nutrient uptake (this chapter) and on citrate exudation (Chapter 3), will reveal zones of the root that are more affected by Al, allowing a qualitative evaluation of the potential role of organic anions, exuded distinctly along the root axis (Chapter 3), in a mechanism of protection against the adverse effects of Al on nutrient uptake.

Materials and methods

Plant material and growth conditions

The plant material consisted of two maize genotypes differing in resistance to Al. These genotypes, CMS36 (Al resistant) and BR106 (Al sensitive), were selected from a collection of ten Brazilian maize genotypes screened before for resistance to Al in both culture solution and acid soil. These two genotypes showed the greatest difference in resistance to Al among the ten genotypes tested (Chapter 2). Maize seedlings were grown in a standard nutrient solution with pH 4.0 and the following chemical composition (m*M*): 1.0 NH_4NO_3, 0.005 NaH_2PO_4, 0.5 K_2SO_4, 0.5 $CaCl_2$, 0.125 $MgSO_4$, and (μM): 46 B, 0.3 Cu, 286 Fe (as Fe-EDTA), 0.1 Mo, 9.2 Mn, 0.8 Zn. They were grown in a controlled environment chamber at 20 °C under a regime of 16 h light (light intensity 80 W m^{-2})/8 h dark.

Roots of seedlings of the two genotypes were grown axenically to preserve organic compounds exuded by roots and their potential role in protecting the roots against the harmful effects of Al. Thus, all materials used in this experiment were autoclaved and successive treatments were carried out in a laminar-flow hood. To sterilise the surface of the seeds, they were immersed for 1 min in 96% ethanol, soaked for 1 h in a solution containing 1.5% sodium hypochlorite (from commercial bleach) + 1% Tween 20, and subsequently incubated for 15 min in a 1.5% sodium hypochlorite solution. After each treatment the seeds were rinsed three times with sterile demineralised water. To check for eventual microbial contamination surface-sterilised seeds were germinated on nutrient agar plates prepared with a 1 m*M* $CaSO_4$ solution. The plates were placed in a dark chamber at 25 °C for 90 h. After germination, uncontaminated seedlings were individually transferred to Petri dishes (∅ 94 mm) containing 50 mL of sterile nutrient solution. The nutrient solution was adjusted to pH 4.0 with 0.1 *M* HCl and autoclaved before the addition of filter-sterilised Fe-EDTA stock solution. In each Petri dish, the roots of one seedling were grown horizontally oriented whereas the shoot was grown vertically through a small notch made in the edge of the lid. The notch was sealed with lanolin and

the Petri dish wrapped with foil and transferred to the growth chamber where the seedlings were grown for 3 d.

Aluminium treatments and nutrient uptake measurements

Aluminium was added to the sterile nutrient solution without Fe-EDTA to reach concentrations of 0 and 40 μM Al. Aluminium was diluted from a filter-sterilised stock solution containing 10 m*M* $AlCl_3$ and 0.1 m*M* HCl. The Al treatment solutions (50 mL) were applied to the seedlings in the same Petri dishes in which they had been growing during the preceding 3 days, replacing the old nutrient solutions. Seedlings were grown for 24 h in the Al treatment solutions before the uptake measurements started.

The divided-root-chamber technique, described by Ryan et al. (1993) and Delhaize et al. (1993), was used to study sites of nutrient uptake along the longitudinal axis of the whole primary seminal root of the seedlings. After 24 h of Al pre-treatment, seedlings were transferred to large Petri dishes (∅ 145 mm) where they had their roots spatially divided using plastic rings (∅ 13 mm). These plastic rings were placed all over the main seminal root of each seedling covering it from the root apex to the most basal section of the root. A thin layer of vaseline was used to seal the space between the plastic ring and the bottom of the Petri dish and a layer of agar was poured around each plastic ring to hold it over the root. Each plastic ring isolated either an apical 10-mm or a 13-mm long root section from the rest of the root system forming an individual chamber. An aliquot of 0.5 mL of Al treatment solution was applied to each chamber and with 50 mL of the same treatment solution the rest of the root system was covered. The Petri dish holding the system was closed with a lid to prevent contamination and evaporation of the treatment solutions, wrapped with foil, and transferred to the growth chamber. The solutions enclosed by the rings were collected after 6 h, weighed, and analysed on P, K, Ca, and Mg. The uptake of nutrients was calculated for each root segment enclosed by one plastic ring for the period of 6 h from the volume and change in nutrient concentration of the solutions in the distinct rings. The latter was measured by ICP–AES. At the end of the experiment the number of lateral roots enclosed by each plastic ring on the more basal section of the root was counted under a binocular (3.2 × magnification) and the root segment in each plastic ring was excised and weighed.

Three replicate seedlings per treatment per experiment were prepared and the entire experiment was repeated once. Because the seedlings used did not have roots of the same length, the number of plastic rings used to cover the entire root varied among replicate roots. Hence, the uptake values measured in the plastic rings could not be directly averaged across replicates neither compared between treatments, unless the number of rings used per root was the same. To overcome this problem, we considered each plastic ring as representing a percentage of the total length of the root. Therefore, the total root

length was divided in 100 units (= 100%) and the uptake value of each ring was repeatedly attributed to every unit that such ring represented. Exemplifying, for a seedling in which nine rings were used to cover the whole root, each ring represented eleven units (= 11%) and the total of nine rings completed 100 units. Once every root had the same relative length, the average of every unit (1%) was calculated among the replicates of the treatments.

Results

Characterisation of the root material used

Maize seedlings had one long seminal root derived from the radicle and typically three to five shorter seminal roots at the time they were used for the nutrient uptake measurements. Only the main (longest) seminal root of each seedling was studied. It was a fully developed root about 120 mm in length and presenting short (< 5–6 mm) lateral roots in the more mature part of the root. This part of the root represented about 30% of the total length. The remaining of the root consisted of the root apex and of an intermediate zone, the part between the root apex and the zone with laterals. The apical zone of the root, enclosed by one plastic ring, represented about 15% and the intermediate zone about 55% of the total root length.

Effects of external Al on total root nutrient uptake

The total uptake of Ca, Mg, and K by the main root was calculated by summing the uptake values measured in the distinct plastic rings used to cover the entire root and is presented in Figure 1. Uptake of P is not shown since a net efflux rather than a net uptake was mostly measured with the seedlings of both genotypes. Seedlings grown without added Al absorbed similar amounts of Ca, Mg, and K during the 6-h period studied, with no significant differences between the two maize genotypes (statistics not shown).

Exposure of seedlings of both genotypes to Al reduced the uptake of Ca and Mg, but not that of K, which was actually stimulated by Al. Uptake of K was about 5 to 6 times as high as that of the seedlings not treated with Al. The relatively high variation in K uptake with plants grown in control solutions is due to the fact that some roots showed a net uptake whereas others showed a net efflux of K when the uptake values of the plastic rings covering each root were summed together.

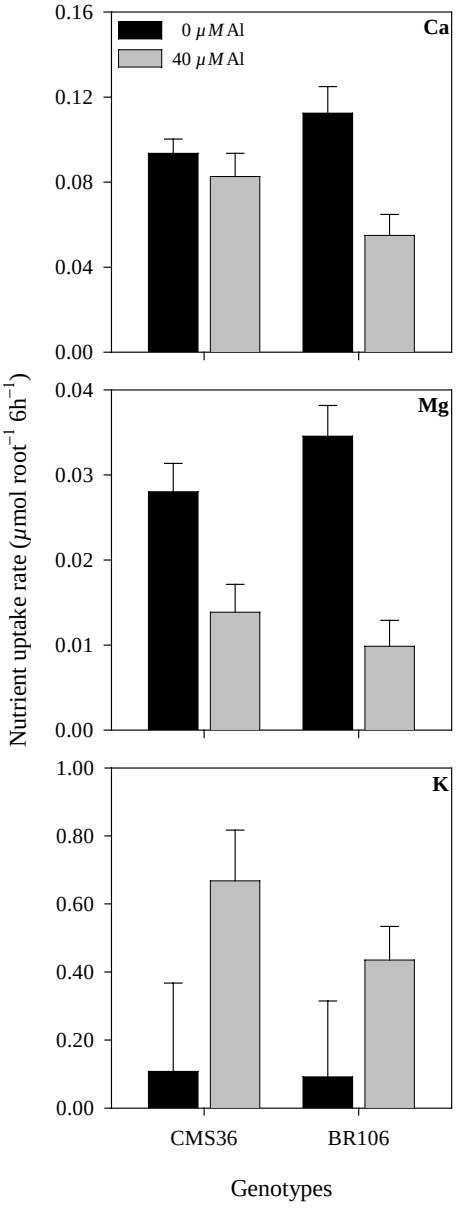

Figure 1. Uptake rates of Ca, Mg, and K by the whole main seminal root of maize genotypes CMS36 (Al resistant) and BR106 (Al sensitive) growing at two concentrations of Al in solution. Total uptake per root was calculated by summing the uptake values measured in the plastic rings used to cover the entire root axis.

The degree of inhibition of Ca and Mg uptake caused by 40 μM Al differed between the Al resistant and the Al sensitive genotype. The negative effects of Al on Ca and Mg uptake were much more pronounced with the Al sensitive genotype BR106. While Al caused a significant reduction of 51% in the Ca uptake of the Al sensitive genotype BR106, the Al resistant CMS36 showed no significant reduction. With the nutrient Mg, both genotypes showed a significant lower uptake after the addition of 40 μM Al. However, the relative Mg uptake of the Al resistant genotype CMS36 maintained about twice that of the Al sensitive BR106 after exposure to Al.

Uptake pattern along the main seminal root

Net fluxes (= influx − efflux) of Ca, Mg, and K were measured along the root axis of the seedlings, regardless of the Al treatment applied. With Ca and Mg mostly a net positive flux (= uptake) was measured along the roots (Figures 2A,C, 3A,C). Similar patterns of nutrient absorption along the root axis were found with the two genotypes when growing in control solutions (0 μM Al), with slightly higher rates of absorption of Ca and Mg in the most basal part of roots of the Al sensitive genotype BR106. This variation was responsible for the higher amounts of Ca and Mg absorbed by BR106 in nutrient solution without Al (Figure 1). With K, neither an uptake nor a net negative flux (= efflux) occurred homogeneously along the root axis; they occurred alternately along the tested roots of both maize genotypes (Figure 4A,C).

Addition of Al to the nutrient solutions generally reduced the uptake rate of the divalent cations Ca and Mg with seedlings of both genotypes, with more severe effects on the Al sensitive BR106 (Figures 2B,D and 3B,D). This negative effect of Al, shown previously on the whole root basis (Figure 1), was again more pronounced with Mg than with Ca.

The effects of Al on nutrient uptake, mainly negative for Ca and Mg, and positive for K, varied enormously along the root axis. They ranged from an increase of 60% in Ca and 15% in Mg uptake to a reduction of 70% in Ca and of more than 100% in Mg uptake. Relative changes (decreases) larger than 100% indicate a complete reversal from uptake to efflux of the nutrient. In general, the smallest reductions in Ca and Mg uptake caused by Al were observed in the intermediate zone, with some variation though. Certainly the strongest negative effects of Al on Mg uptake were observed in the apical region of the root, where a very small uptake of Mg under control solutions (Figure 3A,C) changed into a net leakage of Mg after addition of Al in both genotypes. The positive effects of Al on K uptake were observed in the root zones behind the apical zone, where a consistent K uptake was observed along the root axis. In the apical root zone, however, Al was not able to convert the efflux into an uptake with neither genotype (Figure 4B,D).

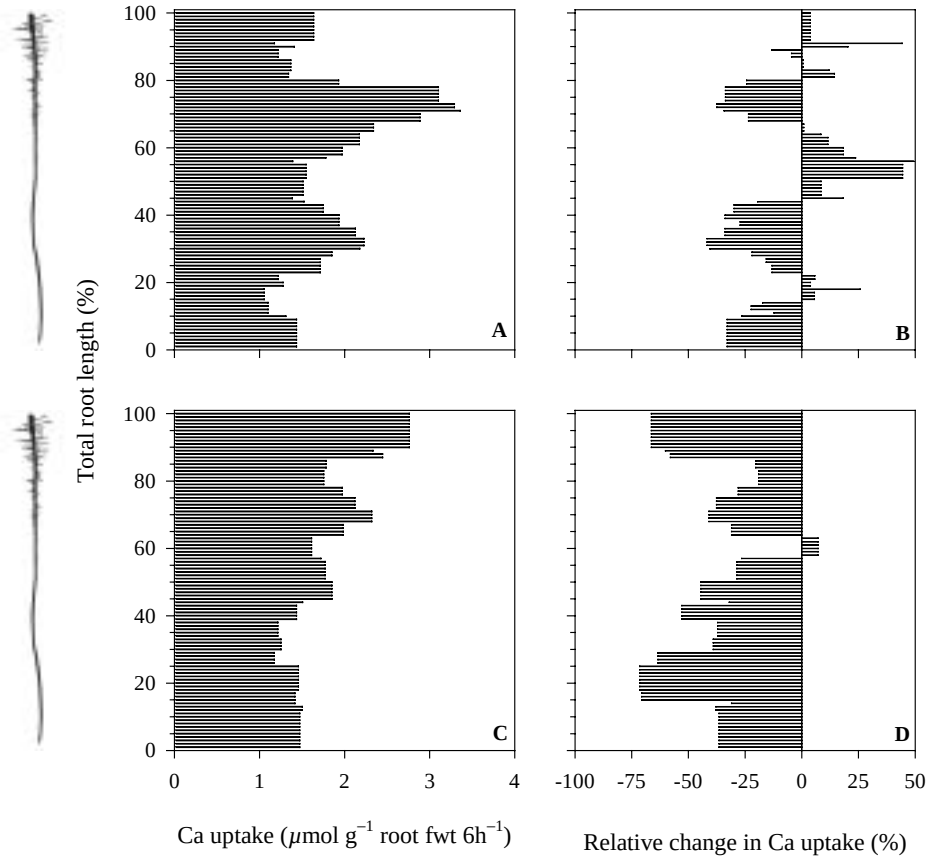

Figure 2. Ca uptake measured along the main seminal root axis of two maize genotypes in control solutions (0 μM Al) [(A) CMS36 and (C) BR106] and respective relative changes caused by 40 μM Al [(B) CMS36 and (D) BR106]. In the right part (B and D) a negative value denotes percentage reduction, while a positive value denotes percentage increase in uptake due to Al exposure. The length of the root axis is expressed in percentage as defined in Materials and methods. Root axes as used in the experiments are schematised on the left-hand side for reference.

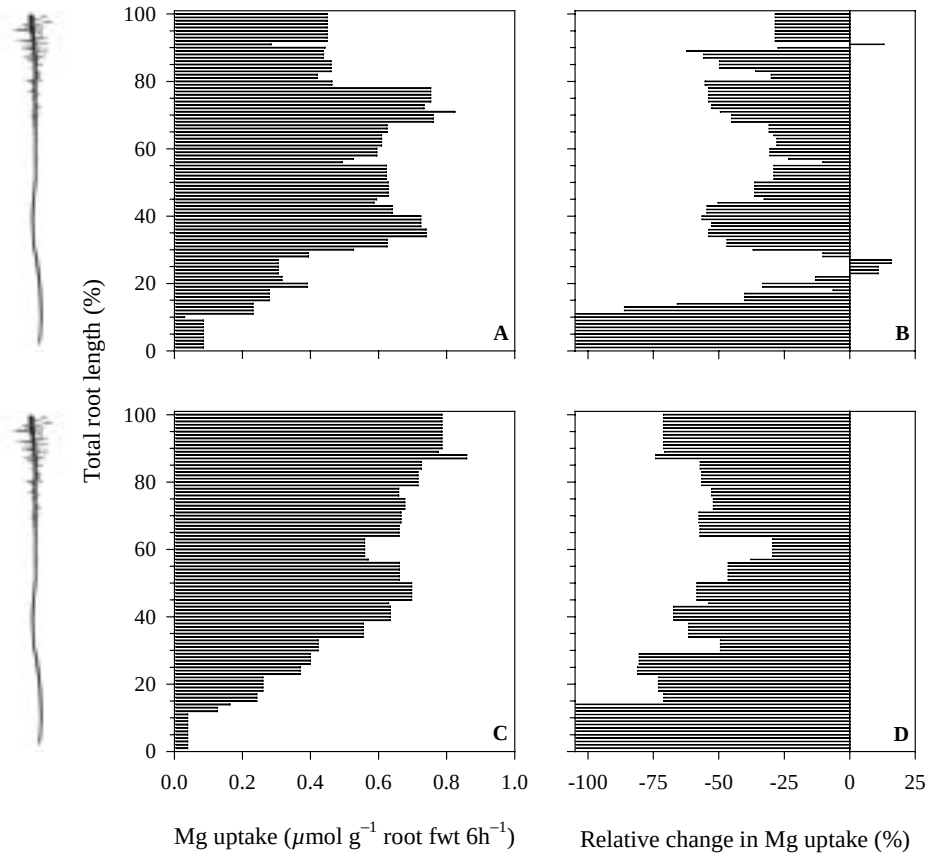

Figure 3. Mg uptake measured along the main seminal root axis of two maize genotypes in control solutions (0 μM Al) [(A) CMS36 and (C) BR106] and respective relative changes caused by 40 μM Al [(B) CMS36 and (D) BR106]. In the right part (B and D) a negative value denotes percentage reduction, while a positive value denotes percentage increase in uptake due to Al exposure. Relative values larger than 100% indicate a complete change in the flux direction. The length of the root axis is expressed in percentage as defined in Materials and methods. Root axes as used in the experiments are schematised on the left-hand side for reference.

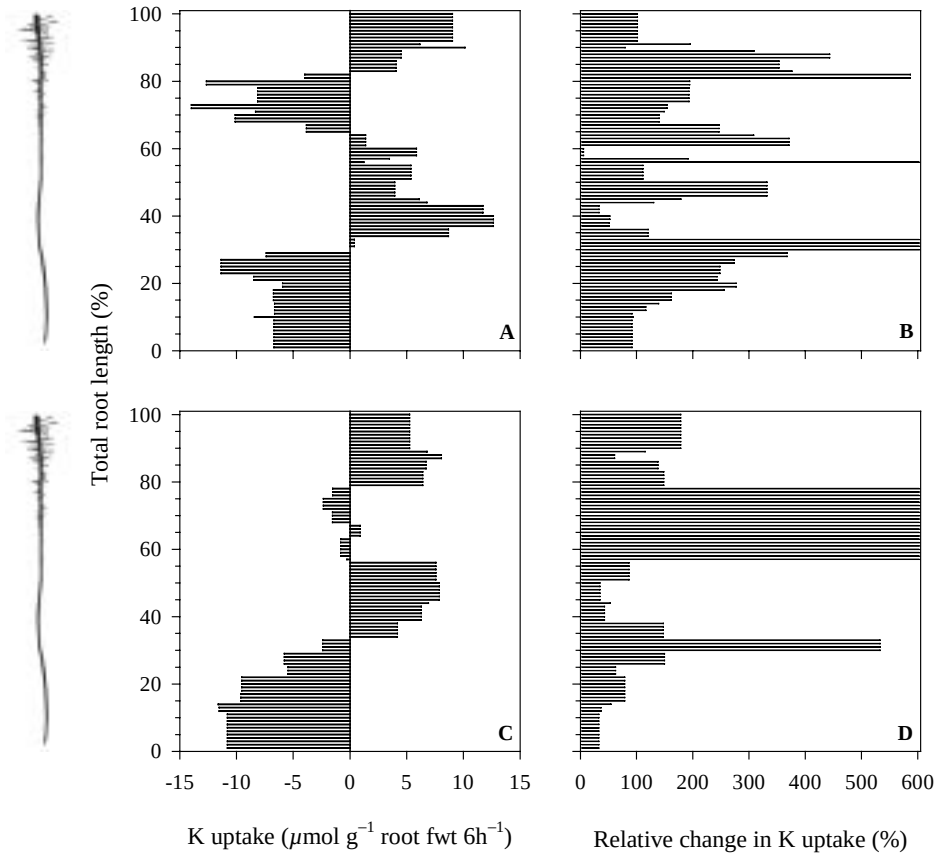

Figure 4. K uptake measured along the main seminal root axis of two maize genotypes in control solutions (0 μM Al) [(A) CMS36 and (C) BR106] and respective relative changes caused by 40 μM Al [(B) CMS36 and (D) BR106]. In the left part (A and C) a positive value denotes an uptake, while a negative value denotes an efflux. In the right part (B and D) a positive value can denote percentage increase in uptake or percentage reduction in efflux or, when larger than 100%, a complete change of efflux into uptake. The length of the root axis is expressed in percentage as defined in Materials and methods. Root axes as used in the experiments are schematised on the left-hand side for reference.

Discussion

The whole surface of the main seminal root of the maize seedlings tested in this study showed activity in absorbing Ca, Mg, and K from the external solution. These results agree with those of other studies with intact roots of maize, barley, marrow, and wheat (Ferguson and Clarkson, 1975; Ferguson and Clarkson, 1976; Harrison-Murray and Clarkson, 1973; Huang et al., 1992). Using a variety of methods to study uptake and release of ions along the roots, these studies indicated that ion absorption capability is widely distributed over the total root surface and not restricted to apical zones of the root.

To avoid significant Al-induced changes in root functioning and therefore the effect of confounding factors on nutrient uptake (Rengel, 1992a), the effects of Al were measured after a short exposure of the root system to this trivalent metal. A total exposure of 30 h revealed strong effects of Al on the uptake of mono and divalent cations with both maize genotypes. Although it is well established that Al impairs uptake of several nutrients, with Ca and Mg being the most affected ones (Marschner, 1995), the mechanisms by which Al disturbs the uptake processes are not well understood. For instance, displacement of the cations Ca^{2+} and Mg^{2+} from exchange sites in the root apoplast by Al has been shown in a number of studies (Godbold and Jentschke, 1998; Keltjens, 1995; Rengel and Robinson, 1989b), and often found to be associated with reduced Ca and Mg uptake and transport to the shoots. But despite some speculation that altered concentrations of nutrients in the vicinity of postulated membrane transport proteins may affect nutrient transport across the plasma membrane (Rengel and Robinson, 1989b), studies have failed to show a functional relationship between adsorption of nutrients in the root apoplast and their absorption across the plasma membrane (Huang et al., 1992). It seems likely that Al is affecting distinctly and independently the adsorption of cations at root exchange sites and their absorption across the plasma membrane. This might explain a simultaneous displacement of K from the exchange sites without changing, or even increasing, as shown here, the absorption of K by the root. It has been also proposed that Al affects uptake at the plasma membrane level, competing for uptake on carriers sites or inactivating these membrane transporters (Taylor, 1988). Studying the effects of Al^{3+} on the kinetics of Mg^{2+} uptake into root cells of ryegrass, Rengel and Robinson (1989b) reported that an Al^{3+} activity of 6.6 μM considerably increased the Michaelis constant (K_m), but not the maximal influx rate (I_{max}), of net Mg^{2+} uptake. Increased K_m indicates that the postulated transport proteins in the membrane have a lower affinity for Mg^{2+} than for Al^{3+} and the authors suggested a mechanism of competitive inhibition to explain the reduced uptake of Mg in the presence of Al. Preferential binding of Al to the plasma

membrane can neutralise its surface charge or even cause a surface potential shift to more positive values, with direct implications on ion movement towards the sites of absorption (Ahn et al., 2002; Ryan and Kochian, 1993). Additionally, Huang et al. (1992) suggested that the mechanism of Al inhibition of Ca uptake probably involves blockage of a Ca^{2+} channel in the root cell plasma membrane by an interaction of Al with the channel at the outer face of the membrane. The higher depression in Mg^{2+} than in Ca^{2+} uptake is presumably because the highly hydrated Mg^{2+} ion has a lower binding strength at the exchange sites on the cell walls and plasma membrane than Ca^{2+}, suffering more from the competition with other cations of higher valency and higher affinity for binding sites.

Results of the detailed experiments with root axis showed that Al is affecting nutrient uptake widely along the main seminal root axis. Although strong effects were noted in the apical part of the root, especially for Mg, reductions observed in more mature zones will have consequences to the overall nutrition of the plant. Root zones other than the root tip are undoubtedly essential and very important in absorption and translocation of several nutrients to the shoots. Huang and co-workers (1993) showed that 75% of the total Ca transported to the shoots of wheat seedlings was absorbed by root regions that were not apical. Moreover, nutrients absorbed in more mature zones of the root are generally more translocated to the shoots (Burley et al., 1970; Ferguson and Clarkson, 1976).

Differential resistance to Al concerning nutrient uptake was observed with the two maize genotypes tested, where the Al resistant CMS36 maintained relatively higher rates of uptake than the Al sensitive BR106. This differential behaviour observed here agrees with the differences in their general resistance to Al, established before with a series of screening experiments based mainly on root growth (Chapter 2). For instance, grown for 4 days at 40 μM Al, the same Al concentration as used here, the Al resistant CMS36 had a reduction of 26% in the root elongation rate (RER), whereas the Al sensitive BR106 had a reduction of 60% in RER. Exudation of organic anions by roots in response to toxic monomeric Al was then cited as the mechanism underlying these genotypic differences. High rates of root citrate exudation measured with CMS36 were mentioned as possibly responsible for its less disturbed root elongation and thus higher resistance to Al compared to BR106, which showed a significant lower rate of citrate exudation. This greater amount of citrate being exuded into the root apoplast and rhizosphere of CMS36 genotype could also mean protection of the roots from the negative effects of Al on nutrient uptake, which would in turn, contribute to its higher relative resistance to Al. Present in a less charged or electroneutral complex, Al will no longer be adsorbed in great amounts at root exchange sites and consequently no longer immobilise P in the root, nor compete with Mg, Ca, and K for common absorption sites. As a result, less Al will ac-

cumulate in the root and plant nutrient uptake will be less inhibited than with an equivalent supply of soluble inorganic monomeric Al (Keltjens, 1995).

But to protect the roots from the adverse effects of Al on nutrient uptake, organic anions, and in the particular case citrate, should be released in root regions where high rates of nutrient absorption are taking place. Results of this study on the spatial distribution of nutrient uptake along the seminal root axis and of a previous study on the citrate exudation along identical roots of genotype CMS36 (Chapter 3) were summarised in Figure 5, and show that the two patterns do not match. Actually they are quite distinct from each other since the highest rates of citrate exudation were observed in root regions where uptake of Mg was fairly the lowest. This disagreement is particularly noticeable in the apical part of the root (initial 10% of the root length). Although accounting for 62% of the total citrate exuded by the whole root, the apical part absorbed no more than 3% of the total Mg (8% of the total Ca) taken up. The root is therefore exuding most of its citrate from a region where the Mg uptake is almost zero.

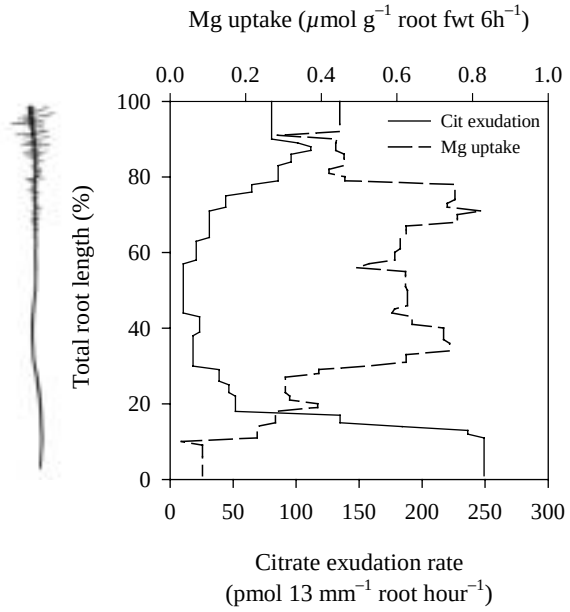

Figure 5. Exudation rates of citrate and uptake rates of Mg measured along the main root axis of the Al resistant maize genotype CMS36. The measurements were done in two separate experiments. Citrate exudation was measured in roots grown at 40 μM Al whereas Mg uptake was measured in roots grown in control solutions (0 μM Al). The length of the root axis is expressed in percentage as defined in Materials and methods. A root axis as used in the experiments is schematised on the left-hand side for reference.

From this we conclude that citrate is probably primarily involved in making plants resistant to Al by detoxifying Al around the root meristems, the most sensitive part for root growth. Based on the findings as presented in Figure 5, local citrate exudation does not seem to be directly involved in nutrient uptake, because the segment with the highest citrate exudation rate (the apex) shows almost no nutrient uptake, while the root zone with the highest nutrient uptake shows almost zero citrate exudation. However, with plants grown in soil systems the intermediate root segment, i.e. the zone with almost no citrate exudation, can probably benefit from the citrate exuded before by the root apex. This citrate can probably protect the intermediate root zone against Al when following the apex through a citrate-enriched soil environment. This could explain the positive role of citrate, exuded by the apex, in nutrient uptake under acid soil conditions. Maintaining the uptake of nutrients on a higher rate is probably a secondary and additional effect of root citrate exudation to make plants more resistant to Al. Genetically, it appears that Al resistance in maize is a complex trait (Magnavaca et al., 1987), and it should not be ruled out the possibility of other mechanisms of Al resistance operating concurrently within this crop species.

Acknowledgements

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Chapter 7

Simulation of citrate exudation into the apoplast of maize roots and the local complexation of aluminium[♣]

Abstract – The interest for the involvement of organic anions in plant aluminium (Al) toxicity/resistance mechanisms has increased enormously in the last years. Released by roots exposed to Al, these organic anions are thought to reduce Al activity locally in the root apoplast and/or rhizosphere to confer protection to root cells against the toxic effects of Al ions. Recent studies on the physiology of root organic anion exudation in response to Al toxicity have strongly supported their role in this mechanism. But it has also raised the question whether quantitatively the amounts of these small ligands released in the root environment are adequate to explain resistance to Al. In the present study this question was tackled by combining experimental with modelling work. We hypothesised that resistance to Al is achieved via modification of the apoplastic environment to effectively protect cell wall and cell membrane function from toxic Al. We used a mechanistic model to describe the dynamic build-up of citrate concentrations in the root apoplast and the citrate diffusion to the outer root medium, and confronted the final outcome of the calculations with observed experimental data. The citrate potential to complex Al at local conditions was predicted with a detailed chemical speciation model. The model calculations suggest that Al activity in the apoplastic compartment can only be kept lower than in the (soil) solution in a dynamic system, where a continuous efflux of a complex forming agent exists. The results strongly supported the notion that citrate can underlie Al resistance in maize. For the conditions considered in this study, detoxification of apoplastic Al and protection of this compartment seem more realistic and more important than those in the interface root-outer solution. A careful extrapolation of how the processes might work in soil systems indicated an even higher potential of this mechanism of protection of roots against Al in soils. Important factors that might well strengthen the role of organic anions are discussed.

Key words: aluminium resistance, aluminium toxicity, modelling, organic anions, rhizosphere, *Zea mays*

[♣] with Willem H. van Riemsdijk, Johannes C. L. Meeussen, and Willem G. Keltjens

Introduction

Aluminium (Al) ions are toxic to plants. Primarily they restrict root growth but, after sufficient exposure, a multitude of symptoms of Al toxicity appear on both roots and shoots (Foy, 1984). Inhibition of root growth, however, occurs only when Al interacts with cellular components of the root apex, whereas application of Al to differentiated regions of the roots has no effect on growth (Ryan et al., 1993). Coincidentally, it is in the apical part of the root and its meristems that Al ions accumulate at highest concentrations. Within the root apex Al accumulates in the apoplast of epidermal and cortical cells, with the endodermis acting as a distinctive barrier to the further penetration of Al into the stele (Rengel, 1996). Although it remains unresolved whether Al uptake across the root cell plasma membrane is a necessary prerequisite for Al toxicity (Rengel, 1996; Taylor, 1995), it seems clear that Al toxicity is expressed in the epidermal and cortical root cells.

Release of metal chelators by roots of plants suffering from Al toxicity has been considered as a possible mechanism underlying plant resistance to Al. Among the metal chelators detected in root exudates of plants growing under Al stress, organic anions of low molecular weight have certainly constituted an important group. Consequently the interest for their involvement in Al toxicity/resistance mechanisms has increased enormously in the last years (Jones, 1998; Kochian, 1995; Ryan et al., 2001). Hypothetically, organic anions released by roots in response to Al confer protection to the roots by complexing Al in the root apoplast and rhizosphere, thus reducing Al activity and toxicity at local target sites such as cell walls and cell membranes (Delhaize and Ryan, 1995; Jones, 1998; Suhayda and Haug, 1986). This mechanism has also been referred to as an exclusion mechanism, where Al is prevented from crossing the plasma membrane, entering the symplast, and reaching sensitive intracellular sites (Taylor, 1991).

Recent studies on the physiology of root organic anion exudation in response to Al toxicity have undoubtedly revealed important features of processes implicated in such a mechanism (for a review see Ryan et al., 2001). However, these findings have been mostly qualitative and a more quantitative evaluation of the role played by organic anions in such a mechanism is lacking. For example, it remains to be shown that the observed fluxes of organic anions are sufficient to protect root apices from toxic Al species. The use of modelling techniques appears in this context a useful tool to complement such studies and to provide insight into the mechanistic basis of some processes involved.

In the present study we have investigated the dynamic of the build-up of citrate concentrations in the root apoplast and the citrate diffusion to the outer root medium. Citrate

release by roots in response to a number of environmental stresses (e.g. Al toxicity, and phosphorus and iron deficiency) has been well documented. Citrate appears to be the primary organic anion released by roots of maize (*Zea mays* L.) plants grown under Al stress conditions, representing as much as about 70% of the total organic anions exuded under such conditions (Chapter 3; Jorge and Arruda, 1997; Pellet et al., 1995). Furthermore, citrate is a well-known strong metal chelator and might well account for the reduction of the negative effects of Al ions on roots. Based on experimental data collected in a previous study (Chapter 3) we aimed to describe part of the processes of citrate release by roots of maize. We used the observed experimental results as a reference to the final outcome of our calculations. The complexation of Al at local apoplastic conditions was also studied. We have focused on the hypothesis that resistance to Al is achieved via modification of the apoplastic environment to effectively protect cell wall and cell membrane structure from Al ions. It is also hypothesised that distinct exudation rates as measured in our experiments with two maize genotypes might be underlying the observed genotypic variation in resistance to Al. Moreover we expect that the results of this study will indicate the effectiveness and possible restrictions of root organic anion exudation as a resistance mechanism to Al.

The mechanistic model describing this system combines a model of citrate release by root cells and diffusive transport of citrate and Al species in solution with a detailed model of chemical speciation. Formation of Al complexes has a strong effect on the activity of free Al^{3+} and consequently on Al phytotoxicity (Hue et al., 1986; Keltjens, 1995; Kinraide, 1991). But the Al^{3+} activity in the root can only be kept lower than in the (soil) solution in a dynamic system, where a continuous efflux of complex forming agents might keep the Al^{3+} activity locally lower than in solution. Given the enormous source of Al that the (soil) solution may represent, sufficiently high concentrations of these complex forming agents at critical sites seem highly necessary.

Adsorption of Al to cation exchange sites of cell walls, although widely discussed in the Al toxicity phenomenon, is not included in the calculations in this stage. Although inclusion of ion exchange processes is in our model framework technically relatively simple, it is in our opinion not a crucial process for alleviating Al stress. The ion exchange process will certainly lead to a retardation of the penetration of Al into the root, due to cation exchange with bound Ca while Al is moving into the root. However, once the exchange sites are in equilibrium with the solution phase of the intercellular space of the root it has no longer an effect on the Al activity in the root. We think therefore that the ion exchange mechanism cannot explain the relief of Al stress.

It may appear that release of organic anions by roots and their subsequent accumulation on the outer root surface would create a chemical barrier to the radial penetration of Al into the root epidermis/cortex. However, the diffusional transport of Al from the outer

solution into the root will not be affected by a concentration gradient of small ligands that can form complexes with Al around the root if it is assumed that the diffusion coefficient for all Al species is the same. Independently of the presence of this barrier formed by small ligands the total amount of Al entering the root apoplast will be the same. The concentration of Al within the root apoplast will be ultimately and solely determined by the Al concentration in the (soil) solution, even if all cation exchange to the cell wall and cell membrane is taken into account. More important is the speciation of the Al entering the root apoplast. It may vary considerably depending on the presence and magnitude of such chemical barrier, and physiologically, this may become of primary importance.

System description

The system studied here is composed of a root segment of 10 mm in length standing in the middle of a plastic ring of 10 mm in height (Figure 1). The impermeable plastic ring is filled with pure nutrient solution thus forming a cylindrical volume around the root segment. The experimental set-up used to collect information on part of the processes to be described here is fully described in Chapter 3. One can immediately see that in the experiments described in Chapter 3 the plastic ring was placed around the root differently from that described above. In the experiments the plastic ring was placed over a root lying on the bottom of a Petri dish. Nevertheless, to simplify the simulation, in the present study the root segment was considered as placed vertically in the middle of the plastic ring. Because of this change in the position of the plastic ring around the root segment and to keep the ratio of volume of nutrient solution to root length the same as used in the experiments, the radius of the virtual plastic ring was adjusted. In the simulation model a plastic ring of 10 mm in height and 4 mm in radius holds 0.5 mL of nutrient solution around a root segment of 10 mm in length and 0.44 mm in radius.

This root segment was chosen to represent the apical 10 mm of a typical main seminal root of maize. On the longitudinal axis this root segment is considered as a homogeneous cylinder without root hairs. Internally two compartments are distinguished: 1) an inner cylinder representing the stele and the endodermis, and 2) an outer cylinder representing the root cortex and epidermis. The inner compartment is considered as an impenetrable hollow cylinder and thus inactive in the processes studied here. The root epidermis and cortex, formed by a series of well-organised concentric layers of cells, are represented in the model by concentric layers of about one cortical cell thickness (Figure 1). Thus the root epidermis/cortex compartment was radially divided into 8 layers, each of 30.25 μm thickness, resembling the mean 8 epidermal/cortical cell layers found in the apical part of a young maize root (Peterson et al., 1993). The cylinder of solution sur-

rounding the root segment was also divided into several concentric layers, where we used the numerical solution of the diffusion process.

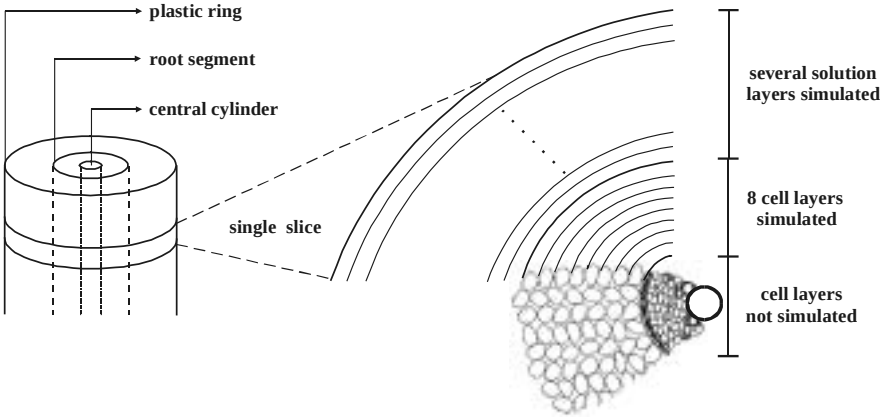

Figure 1. Schematic representation of the simulated system.

The chemical composition of the solution used in the calculations is actually a simplification of the nutrient solution used in the experiments of Chapter 3 in the sense that only the major components, also present in higher concentrations, were considered. This solution consisted of the following components, with respective concentrations in μM: calcium (Ca), 500; chloride (Cl), 1000–1300; sulphate (SO_4), 625; aluminium (Al), 0–100. The pH of the solution was 4.0.

Model description

Chemical speciation

The chemical speciation model calculates the distribution of the components among their various species in solution. Surface reactions describing interactions of the chemical species with root cell walls and root cell membranes are not taken into account in the model for reasons explained in the introduction. The chemical speciation of the system during transport is computed from the local total component concentrations. However, it is also possible to compute the speciation at a fixed pH when desired. Because of the sensitivity of the speciation results to the equilibrium constants, the values used in the calculations are listed in Table 1.

Transport model

The movement of components in the solution inside and outside the root is described by diffusion. Only radial diffusion is considered and it is described using Fick's second diffusion law (Crank, 1975):

$$\frac{\partial C_i}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left(rD\frac{\partial C_i}{\partial r} \right)$$

where C_i stands for the component concentration in mol m^{-3}, t for the time in seconds, r for the radial distance from the centre of the layer in meters, and D for the apparent diffusion coefficient in $m^2\ s^{-1}$. No distinction in the diffusion coefficient of species was made. A single value of $1 \times 10^{-9}\ m^2\ s^{-1}$ (self-diffusion coefficient in water at infinite dilution) was used for all species in the solution outside the root. Slower diffusion of ions in the apoplast of plant roots than in free water was simulated by using a smaller diffusion coefficient ($D = 1 \times 10^{-10}\ m^2\ s^{-1}$) for the solution in the root, as suggested by Mengel and Kirkby (1987). Slower diffusion of ions in this compartment than in free water is probably due to the tortuosity and to the interactions of ions with the cell walls.

Because we studied exudation of citrate in a closed system and for a relatively short time period we assume root absorption of water to be negligible in this period. Consequently, no convective transport driven by water movement in this system was taken into account. Mass exchange between the solution in and outside the root is assumed to be controlled by diffusion only and not to be restrained by the permeability of the outer root surface or by a boundary layer.

The combined set of equations that describe the mass exchange in the system is numerically implemented by using a one dimensional so-called mixing cell model and solved by using a first order difference scheme in time (van Beinum et al., 1999). At every time step, first all the potential mass exchanges were calculated for every cell in the system, before the mass totals were actually updated. The distribution of the components in solution is computed each time step using the speciation model. The simulations were carried out using a time step of 0.1 s. Increasing the number of time steps and layers (nodes) did not change the calculated results noticeably, indicating that the results are not significantly affected by numerical errors.

Table 1. Equilibrium constants used to compute speciation in solution

Equilibrium reaction			Log K
$Al^{3+} + OH^-$	↔	$AlOH^{2+}$	8.98
$Al^{3+} + 2OH^-$	↔	$Al(OH)_2^+$	18.70
$Al^{3+} + 3OH^-$	↔	$Al(OH)_3$	27.01
$Al^{3+} + 4OH^-$	↔	$Al(OH)_4^-$	32.67
$Al^{3+} + Cit^{3-}$	↔	AlCit	7.37
$Al^{3+} + 2Cit^{3-}$	↔	$AlCit_2^{3-}$	13.90
$Al^{3+} + SO_4^{2-}$	↔	$AlSO_4^+$	3.20
$Al^{3+} + 2SO_4^{2-}$	↔	$Al(SO_4)_2^-$	5.10
$Cit^{3-} + H^+$	↔	$HCit^{2-}$	6.40
$Cit^{3-} + 2H^+$	↔	H_2Cit^-	11.20
$Cit^{3-} + 3H^+$	↔	H_3Cit	14.30
$Ca^{2+} + Cit^{3-}$	↔	$CaCit^-$	4.70
$Ca^{2+} + Cit^{3-} + H^+$	↔	CaHCit	9.50
$Ca^{2+} + Cit^{3-} + 2H^+$	↔	CaH_2Cit^+	12.30
$Ca^{2+} + OH^-$	↔	$CaOH^+$	1.30
$Ca^{2+} + 2OH^-$	↔	$Ca(OH)_2$	0.01
$Ca^{2+} + SO_4^{2-}$	↔	$CaSO_4$	2.31
$SO_4^{2-} + H^+$	↔	HSO_4^-	1.98

Model calculations

The present numerical model simulates release of citrate into the solution-filled intercellular spaces of the root epidermis and cortex (= root apoplast). According to Marschner (1995), the root apoplast represents about 5–10% of the total root volume. Therefore, to account for this reduced volume, where the diffusion process takes place, the layers representing the root epidermis/cortex had their volume proportionally reduced to account for 5% of the total volume of the root segment. Because mass exchange between two neighbouring cells (layers) is calculated in the model by multiplying the flux with the contact area between the neighbouring cells, it is necessary to reduce the surface contact area accordingly. Along the longitudinal axis, the effect of varying the portion of the root segment that supposedly releases citrate is studied with the model.

This is done because of speculations in the literature that probably only the first few mm of the root tip are responsible for the release of citrate in response to Al.

The radial location of citrate release in the root epidermis/cortex is also unknown. Therefore, in the simulations the release of citrate is assumed to take place at different radial positions (cell layers) in this region of the root. The rates of citrate release used in these simulations were calibrated using values of citrate exudation measured in the experiments described in Chapter 3. The mean concentration of citrate measured in the bathing solution of the root at the end of 4 h of exudation was simulated assuming a constant rate of release that was calibrated using the experimental data and the model. As also shown by the experimental studies, exudation rates vary according to the Al concentration in solution.

It is assumed that initially neither the root apoplast nor the outside solution contain any citrate, and that the solution in and outside root contains the same total Al concentration. The exudation of citrate is triggered at time zero (t_0). Calculations were performed at a fixed pH of 4.0. The implementation of the chemical speciation model with the transport model is done with a recently developed object oriented framework for reactive transport modelling [Objects Representing CHEmical Speciation and TRAnsport (ORCHESTRA)] (Meeussen, 2003).

Results and discussion

Al speciation in the outer root solution

Before calculating citrate release into the apoplast of epidermal and cortical root cells and its subsequent diffusion to the outer root medium we calculated speciation of Al in the solution surrounding the root segment. In this calculation we took the highest citrate concentration in the bathing solution found in the experiments of Chapter 3. A mean concentration of 2 μM citrate was measured in the bathing solution at the end of a 4-h exudation period for roots exposed to 40 μM Al. As expected, this concentration of citrate was too low to complex a significant fraction of the toxic Al^{3+} species present. At pH 4.0 and an Al:citrate molar ratio of 20, Al-citrate complexes accounted for less than 1% of the total Al whereas the major part of it (67%) remained as the trivalent cation Al^{3+}.

We used a citrate concentration that represented the average concentration in solution and one might argue that it could be higher at the outer surface of the root since the solution around the root remained unstirred during the exudation period. Nevertheless, we do not expect significantly higher concentrations at the root surface, simply because the outside medium was a pure aqueous solution in which ions could freely diffuse away

from the root. Predicted concentrations of citrate at the root surface will be shown and discussed later in this chapter.

Citrate concentration in the root apoplast

We therefore started investigating the release and accumulation of citrate in the relatively small volume of the root apoplast, where citrate would presumably be most effective in detoxifying Al. But according to the calculations shown above, relatively high concentrations of this metal chelator should be present, in this case in the apoplast, in order to complex a major part of Al^{3+} and thus confer protection to sensitive sites. To test this hypothesis we set a citrate concentration to the apoplast that would lower the local Al^{3+} concentration to an arbitrary value of 50% of the Al^{3+} concentration present in the solution outside the root apoplast. The citrate concentration estimated was 200 μM citrate (Al:citrate molar ratio of 0.2). This concentration of citrate was kept constant in the apoplast throughout a simulated 4-h exudation period. The big concentration gradient between the root and the outer solution caused diffusional transport of citrate out of the root, creating a concentration profile at the outer surface of the root and leading to large amounts of citrate being exuded into the solution outside the root (Figure 2A). After 4 h of exudation the predicted amount of citrate that had left the root was about 20 times as much as that measured in the experiments. This result indicates that by simple diffusion, concentrations of citrate that may not be considered exceptionally high may lead to high amounts of exudation in short time periods. This predicted result therefore does not agree with the observed one and led us to search for possible explanations for this disagreement. This result might be indicative that not the whole 10-mm long root segment (*i*) has similar concentrations of citrate in its apoplast and/or (*ii*) contributes evenly to the exudation of citrate in response to Al, as it was assumed in these calculations.

On the other extreme, as presented in Figure 2B, a relatively low constant apoplastic concentration of 10.6 μM citrate was calculated to be required to drive diffusion of citrate to the solution that would, in 4 h, result in amounts of citrate release similar to that measured experimentally. However, similarly to what has been discussed before, this is too low to complex a significant part of Al^{3+} present in the outer solution. The citrate concentration profile in solution predicted for such conditions is presented in Figure 2B and it shows that the concentration of citrate at the interface root-external solution is only slightly higher than in the bulk solution.

Constant production of citrate by root cells and constant release into the intercellular spaces of the root epidermis and cortex was also evaluated in this study, in contrast to the constant citrate concentration assumed before. To study the build-up and development of concentration profiles of citrate, a certain rate of this metal ligand entering the root apoplast (i.e. root cell exudation) was imposed as the driving force. In the first set of

calculations we considered only results that correspond with measured citrate exudation at 40 μM Al. The exudation rate used was such that at the end of a simulated 4-h exudation period the total amount of citrate in the outer root solution matched that measured in the previous experiments. This exudation rate was kept constant during the calculations and was assumed to take place at different cell layers of the root epidermis/cortex. In situation I citrate entered the apoplast via the innermost layer of the root cortex, closest to the impermeable central cylinder. Just contrary, in situation II all citrate entered the system at the outermost layer of cells of the root, closest to the outer root solution. In situation III, an equal amount of citrate entered every layer of the root epidermis/cortex.

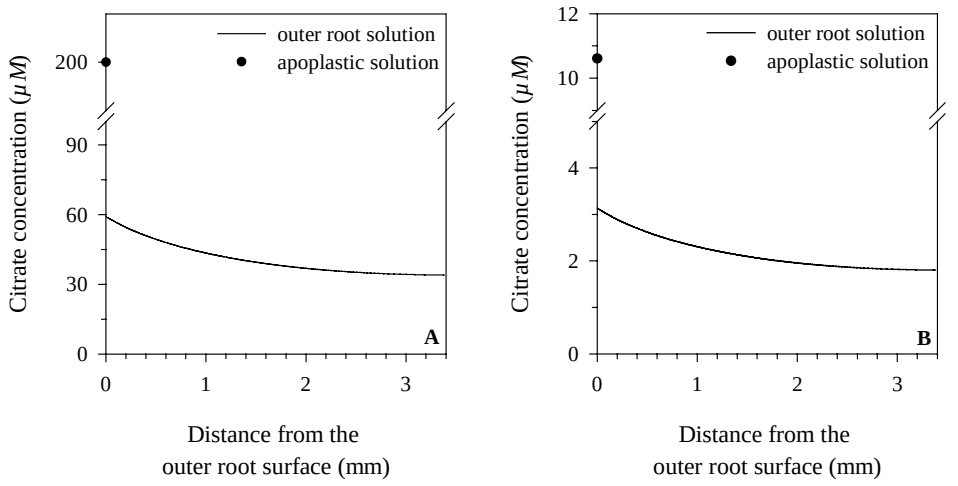

Figure 2. Predicted concentration profiles of citrate in the outer root solution with a constant concentration of (A) 200 μM and (B) 10.6 μM citrate in the apoplast during a simulated exudation period of 4 h.

Although an equal amount of citrate entered the root apoplast in these three situations (equal total exudation rates), a remarkable and significant difference in the predicted concentration profiles of citrate in the root apoplast was observed. These concentration profiles as well as those in the solution outside the root are presented in Figure 3. Regardless of the localisation of citrate release, the predicted profiles developed in the outer root solution were almost identical (Figure 3B). If we assume that citrate is continuously released in the innermost layer of the root cortex, diffusion of citrate to the outer cell layers and external solution creates a steep concentration profile of citrate in the root

apoplast (Figure 3A). Although the concentration of citrate in the whole apoplast increases with time, the highest concentration is reached at the place of exudation (i.e. innermost cell layer). After 4 hours the predicted concentration built-up in the innermost layer was 155 μM, a concentration that is about 13 times that reached in the outermost root layer.

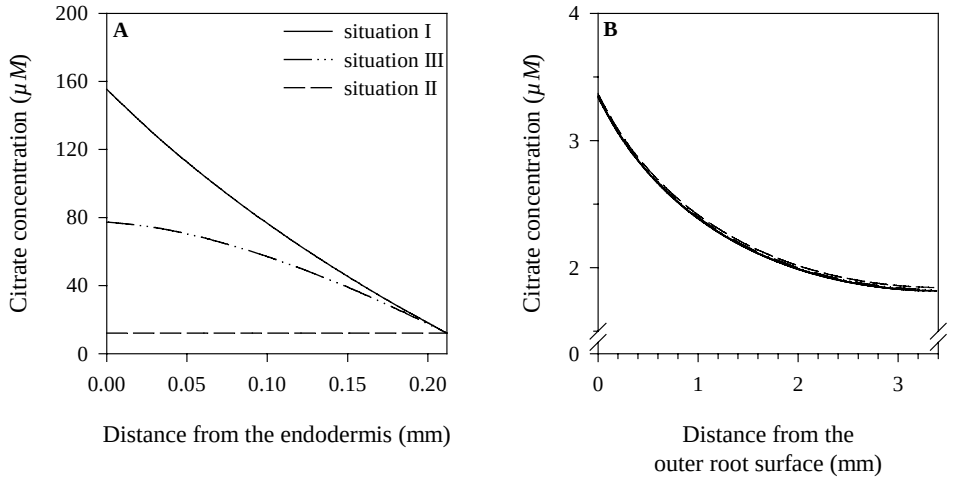

Figure 3. Predicted concentration profiles of citrate (A) in the root apoplast and (B) in the outer root solution with exudation of citrate taking place at three distinct radial positions in the root epidermis/cortex. Exudation rate of citrate corresponding with citrate exudation at 40 μM Al for a total time period of 4 h.

Interestingly, release of citrate only at the periphery of the root (situation II) resulted in a rather distinct citrate status in the root apoplast. Diffusion of citrate towards the centre of the root resulted in an initial gradient profile in the root apoplast, but because of the small distance between the source of citrate and the impermeable central cylinder, this gradient disappeared within a few minutes. Continuous release of citrate into this layer led to a rather homogeneous increase in the concentration of citrate across the cell layers of the apoplast with time. This concentration reached 12 μM after 4 h, thus similar to the lowest concentration reached in the root in situation I. Release of citrate by all 8 cell layers, which assumes that all cells of the root epidermis/cortex possess the mechanism controlling citrate release, also led to a concentration profile of citrate in the root apoplast. Apart from the outer most layer, the predicted concentrations were intermedi-

ate between those predicted in situations I and II. The assessment of the radial localisation of citrate release in the root epidermis/cortex indicates that most citrate will be kept in the root if only the innermost layer of the root cortex is responsible for its release. Consequently, independently of the localisation of the sensitive sites, the highest protective effects are to be conferred by citrate in situation I.

Al speciation in the root apoplast

Assuming that citrate concentration profiles indeed exist in the apoplast of root epidermis and cortex, corresponding concentration profiles of Al species will also be found in this compartment of the root. The steepest gradients in concentrations of chemical species are therefore to be found in the case where citrate is released by cells at the innermost layer of the cortex (Figure 3, situation I). Predicted profiles of different Al species, developed as results of such citrate exudation are presented in Figure 4. Except for Al^{3+}, the different Al species were grouped and presented according to the Al-anion complexes formed. The highest degree of Al^{3+} complexation was predicted for the innermost cell layer of the root where formation of AlCit and $AlCit_2^{3-}$ species reduced the concentration of Al^{3+} by 40%. As a result, Al^{3+} and Al-citrate species accounted each for about 40% of the total Al. Lower values of Al^{3+} complexation were predicted in the direction of the outer root surface, where barely no Al was complexed by citrate. The speciation of Al, as well as of the other components in the apoplast, is assumed to be in dynamic local equilibrium. The dynamic transport process is a kinetic process that changes in time, affecting the local speciation. With an ongoing production of citrate in the root, a concentration profile of citrate in the apoplast (situation I and III), and a concentration gradient between the apoplast and the outer solution (situations I, II, and III) will develop, and will consequently drive diffusional transport of species in and out the root.

Al speciation in the root apoplast versus Al toxicity

Subsequently, speciation of Al in the root was studied in relation to Al toxicity to roots. Complexation of Al at the local apoplastic conditions was evaluated under a variety of total Al concentrations in solution and citrate exudation rates, and related to observed results of Al effects on root growth. For this purpose we used experimental data collected with two maize genotypes [CMS36 (Al resistant) and BR106 (Al sensitive)] in previous studies (Chapters 2 and 3). Citrate exudation rates and Al-induced root growth inhibition measured with these two genotypes exposed to a range of Al concentrations were considered. Due to the different behaviour of the maize genotypes concerning Al-induced root growth inhibition and citrate release, we aimed at finding common values of concentrations of free Al, that once present at allegedly sensitive sites, would lead to similar values of root growth inhibition in both genotypes. For example it could be inter-

esting to show that the extent of the effects of Al on root growth is the same when the activity of toxic Al species at the sensitive sites is the same for both genotypes. For this we would need to assume that the toxic reaction that takes place in the different genotypes causing the negative effect is the same and that other conditions (e.g. pH, competing ions like Ca^{2+}) are also the same. For such analysis we considered that for such conditions Al^{3+} could be used as a reference species controlling phytotoxicity and that its effects on root growth occur at the innermost cell layer of the root cortex. The citrate exudation rates used were adapted so that the predicted citrate released to the outer root solution matched the experimentally measured values. Complexation of Al was calculated at the local conditions predicted for the innermost layer as in situation I (exudation solely at the innermost cell layer).

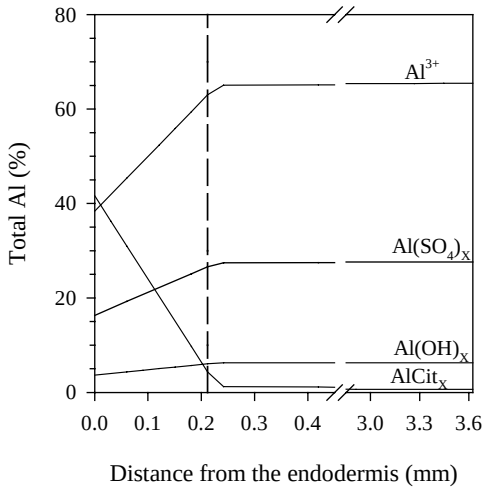

Figure 4. Predicted profiles of Al species expressed as percentage of total Al in the inner and outer root solutions as a result of the citrate concentration profile presented in Figure 3A (situation I). The dashed line represents the interface root-outer solution.

Initially the relative root elongation rate as measured in Chapter 2 was plotted against total Al concentration in solution (Figure 5A), which revealed a noticeable difference in the sensitivity of the genotypes to Al in solution. According to the calculations shown before, it is expected that the different rates of citrate release observed with the two genotypes exposed to various concentrations of Al (Chapter 3) will lead to different status of citrate in the apoplastic compartment, which may in turn significantly affect the

local speciation of Al and its toxic potential. Results of our calculations show that this was indeed the case. Predicted values of toxic Al^{3+} residing in the apoplast of the innermost layer of the root cortex after 4 h of citrate exudation were plotted against relative inhibition of root elongation measured at different degrees of Al stress with both maize genotypes (Figure 5B, C, D). In all cases there was some degree of Al complexation by citrate, which decreased the Al^{3+} activity in the apoplastic solution. Clearly, in all plots lower values of Al^{3+}, which also means higher reductions in Al^{3+} concentration, were predicted for the Al resistant genotype CMS36 than for the Al sensitive BR106. Also, calculated at three increasing values of pH, the complexation of Al by citrate has clearly become higher at higher pH values.

Further examination of Figure 5B, C, D reveals that the values of Al-induced root elongation inhibition start to coincide when plotted against the predicted concentrations of Al^{3+} in the root apoplast. Interestingly, common values of Al^{3+} for similar degrees of root inhibition have arisen from these predictions. In this respect, special attention is given to predicted values that correspond to about 25% reduction in the root elongation rate. At pH 4.5, similar reductions in root elongation rate were caused by a predicted value of about 4 μM Al^{3+}. The shape of these curves, especially those of Figure 5C, also suggests that a certain minimum concentration of Al^{3+}, like a threshold value, must be present before significant negative effects on root growth are observed. Initial total Al concentrations of up to 10 μM for BR106 and up to 20 μM for CMS36 caused either a small reduction or a small increase in the root elongation rate compared with roots grown at control 0 μM Al. The predicted values of free Al^{3+} at pH 4.5 under which no significant reduction was observed were below 3 μM. At concentrations of 3–4 μM free Al^{3+} similar root growth inhibition was observed with both genotypes, while at higher concentrations apparently much higher toxicity occurred and differences between genotypes appeared. The disagreement observed at higher concentrations of Al^{3+} may suggest that the genotypes suffer distinctly from the toxic effects of Al^{3+} at highest concentrations, with much more severe damages on the Al sensitive BR106. It is also suggested that the protective effect of citrate might fail at high Al activity in the system, as follows from Figure 5.

The results of the present study strongly support the notion that organic anions, and in the particular case citrate, released by roots can alleviate the toxic effects of Al ions at sensitive sites of the root. The complexation of Al by citrate may lead to a much lower Al^{3+} activity, which may explain the lower toxicity. As explained in the introduction, a continuous release of a complex forming agent and a dynamic local equilibrium are likely to occur in the apoplast and thus have the potential to explain at least part of the plant's resistance to Al. At least for the conditions considered in this study, detoxification of apoplastic Al and protection of this compartment seem more realistic and more

important than those in the interface root-outer solution. Although the system described here does not represent directly a soil system, where a rhizosphere can be formed and some of the processes may operate quite differently, we should not disregard that a majority of the plant germplasm resistant to Al has been discovered in culture solution systems, where no rhizosphere is formed and detoxification taking place in the apoplast may be playing a major role in the mechanism of resistance.

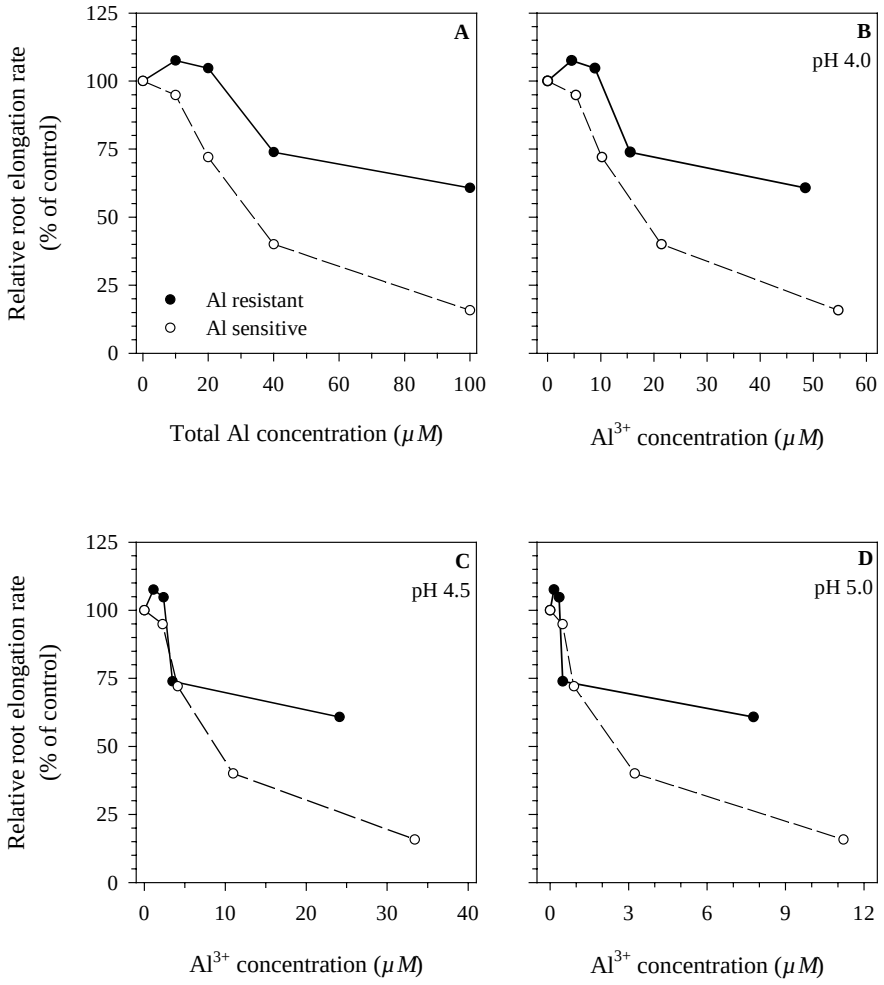

Figure 5. Effect of Al on root elongation of two maize genotypes versus (A) total Al in solution and (B, C, D) free Al^{3+} in the root apoplast at three pH values.

Given our conservative approach with respect to some assumptions made for performing the calculations, we believe in an even greater potential of this mechanism. There are important factors that might well strengthen the role of organic anions in this mechanism. Firstly, it is the portion of the root that is really releasing citrate. The concentrations of citrate predicted for the apoplast are inversely proportional to the length of the root segment releasing citrate. Thus, assuming that only the apical 5 mm of the root are releasing citrate, half of the length considered in the calculations, the concentrations reached in the whole apoplast will be doubled for the simulated time period. Secondly, in a real soil system diffusion of citrate from the root into the soil solution will be slowed due to the presence of a reactive porous medium. Once citrate has left the root it will have a more tortuous and reactive pathway to diffuse through, with adsorption to the soil particles retarding its diffusion and favouring its accumulation around the root (Geelhoed et al., 1999). Thirdly, we have used stability constants of Al-citrate complexes (log K values) that are among the lowest ones reported in literature. For the complex AlCit we have used the value of 7.37 (Lindsay, 1979) whereas values of 7.98, 9.70, and 10.0 have been respectively reported by Nordstrom and May (1989), Blamey et al. (1997), and Motekaitis and Martell (1984). This implies that significantly higher complexation of Al by citrate may be predicted, with direct implications on the effectiveness of citrate to suppress Al^{3+} activity and toxicity.

Finally, it must be clear from this work that root exudation rates of citrate, as experimentally observed, can indeed lead to high amounts of citrate in the apoplast. These citrate concentrations reached can complex a significant fraction of the locally present free Al^{3+} and account for resistance to Al in maize.

Chapter 8

Epilogue

This chapter summarises and discusses the major results of the present study and suggests some directions for future research at the end of each of the following sections.

Selection of maize germplasm for studies on aluminium resistance

The broad, genetically determined differences in the behaviour of plants of the same species when growing under conditions of aluminium (Al) stress may provide clues to mechanisms of Al toxicity and resistance, and aid in plant breeding for superior Al resistance (Foy, 1988). When we screened a collection of ten maize genotypes representing a range of resistance to Al, we were concerned that interactions between the genotype and a particular rooting medium employed might lead to false ratings and originate conflicting results in genotypic differences in resistance to Al. To avoid this problem we carried out screening experiments using different rooting media. We ranked the genotypes for Al resistance based on the results of each experiment, and considered the rankings collectively to select genotypes of significant differential sensitivity to Al to be used in the next studies.

The plant characteristic that best reflected the plant's ability to cope with Al toxicity, on a short-term basis, was either root length itself or variables calculated from measurements of root length in time, i.e. root elongation rate. Genotypic variation in root internal concentration of citrate and malate could not explain the differences in resistance to Al as observed when using root length as indicator. Changes in organic anion concentration were interpreted as the result of Al-induced stress and not as a basis for differential resistance. While we should not rule out that a mechanism of internal detoxification of Al by organic anions (Al tolerance) might exist in maize, in this research, resistance of the maize genotypes to Al was better correlated with root exudation of such organic anions, suggesting that detoxification of Al is probably taking place externally the root or in the root apoplast (Chapter 3). Besides, given the uneven distribution of internal organic anion concentration within a single root axis (Chapter 4; Jones and Darrah, 1995), studies aiming to relate the internal concentration of these solutes with Al resistance should analyse specific regions of the root separately instead of taking the whole root axis or even the whole root system together.

Aspects of root citrate exudation in response to Al exposure

We hypothesised that root release of organic anions constitutes a mechanism of Al resistance in maize. Two previous studies with pairs of maize genotypes had found higher rates of citrate, and to a lesser extent malate, exudation in the more Al resistant genotype than in the less Al resistant one (Jorge and Arruda, 1997; Pellet et al., 1995). But if maize plants are using root exudation of citrate as a mean of protecting their roots from negative effects of Al, then genotypes with greater ability to exude citrate will be more resistant to the effects of Al. With eight maize genotypes out of the ten studied in Chapter 2 we found an exponential-like relationship between citrate exudation rate and root elongation inhibition. While not constituting proof, these results suggested that Al-stimulated citrate release from root apices might account for the resistance exhibited by these genotypes. Further studies proceeded with two genotypes selected to represent the extremes of resistance to Al [CMS36 (Al resistant) and BR106 (Al sensitive)] and addressed aspects of root citrate release in response to Al, like induction period of exudation and spatial localisation of the exudation in the root axis.

Undoubtedly a great achievement was the ability to measure the rate of organic anion release from specific sections of the root for short time periods (4 h). Root exudates were collected from seedlings growing free from microorganisms and for short periods soon after the exudation of organic anions had been induced to higher rates. Dividing intact roots spatially in short segments allowed not only a qualitative evaluation of sites of organic anion exudation but also a quantification of the fluxes of such organic molecules along the main root axis. This experimental set-up allowed us to determine accurately the exudation of organic anions during a period that root growth was minimum and reabsorption of exuded organic anions by the roots can be neglected (Jones and Darrah, 1995).

There is no information on the radial localisation of organic anion release in the root. It was recently reported that both cortical and stellar root cells have the potential to release citrate in the presence of Al (Piñeros et al., 2002). However, the real contribution of these cells to the exuded organic anion observed in experiments remains obscure. Assessment of the radial localisation of organic anion release as well as of the Al sensitive sites in the root will certainly increase our understanding of the mechanistic basis of the processes involved as it was also discussed in Chapter 7.

Quantitative assessment of the role of citrate in protecting the root apex

The results of Chapter 3 automatically raised the question whether the measured rates of citrate and malate release by roots can lead to amounts that are enough to complex a significant fraction of the phytotoxic monomeric Al locally present in the root apoplast and/or rhizosphere. We tackled this question by combining experimental and modelling

work. The use of a mechanistic model to describe part of the processes studied experimentally allowed us to summarise our current knowledge about the processes involved and to discuss the factors that may be crucial in determining the effectiveness of such a mechanism of resistance to Al (Chapter 7). The results strongly supported the notion that citrate can indeed underlie Al resistance in maize. A careful extrapolation of how the processes might work in soil systems is presented in the next section.

An important factor that certainly deserves more attention is the pH of the root apoplast of plants growing in the presence of Al. Measurement of the apoplastic pH of the root cortex, for instance with the use of pH-sensitive electrodes (Felle, 1998), might well provide insight into this important factor directly controlling Al activity in solution and influencing the complexation of Al by organic anions [i.e. pH-dependent complexation (Motekaitis and Martell, 1984)]. In our model calculations the pH was assumed not to change as a result of citrate exudation, which may be untrue in reality. Therefore simulations allowing the pH to vary as a result of exudation of fully protonated or fully dissociated citrate and confrontation of the results with experimental data are recommended.

The potential of citrate released by roots in soil systems

The behaviour of citrate, and other organic anions, in a soil system may differ quite significantly from that in a hydroponics system. In the volume of soil that is influenced by the root (i.e. rhizosphere) a complex of factors may affect the processes controlling the dynamics of citrate. Because these small organic compounds are readily used by soil microorganisms, their quick decomposition in the rhizosphere, a zone of high microbial activity, may become rather important in the soil. Contrary to the relatively rapid diffusion of citrate in pure aqueous solution, diffusion of citrate from the root into the soil solution will be slowed due to the presence of the soil particles. Once citrate has left the root it will have a tortuous and reactive pathway to diffuse through, with adsorption to the soil particles retarding its diffusion and favouring its accumulation around the root (Geelhoed et al., 1999). Adsorption of these organic compounds to the soil particles was also shown to slow down their degradation by soil microorganisms (Jones and Edwards, 1998). Adsorption and protection of these compounds from biodegradation in this zone of high microbial activity was found to prolong their persistence and to enhance their effectiveness in soils (Geelhoed et al., 1999; Jones et al., 1996b).

The relatively slow diffusion in soil will apparently contribute to increase the accumulation and persistence of citrate in the root apoplast, once all the citrate released in this compartment will encounter resistance to leave the root and move through the soil. As predicted with our model calculations in Chapter 7, higher concentrations of citrate will confer an even higher protection to the roots against toxic Al species.

From reducing the excess supply of Al and heavy metals (Mench et al., 1988; Suthipradit et al., 1990; van Hees et al., 2001) to the opposite extreme of enhancing nutrient availability in soil [e.g. mobilisation of phosphorus (P) and iron (Fe) from poorly soluble sources (Geelhoed et al., 1999; Gerke et al., 1994; Hoffland, 1992; Jones et al., 1996a)], a wide range of functions has been ascribed to organic anions in the rhizosphere. Currently, most of these hypothesised processes appear to be beneficial to plants. With respect to Al and Fe the dualistic role of organic anions has in common the formation of a chelate. The presumable positive effect of organic anion with Al is because the metal complex formed is no longer available to the plant, whereas with Fe, the formation of Fe-complexes enhances the Fe mobility in the soil, making it more available to plant roots (Jones et al., 1996a). Apparently contradicting, the difference lies on the type of interaction of these complexes with the root. Dicots and non-grass monocots use a plasma membrane-bound reductase to first reduce Fe^{3+} contained in chelate complexes to Fe^{2+}, before root cells can absorb it. This phenomenon, however, does not seem to occur with Al chelates, which stay in this less reactive form.

Integration of the processes mentioned (i.e. sorption, complexation, and microbial degradation) with fluxes of elements occurring simultaneously in the rhizosphere seems highly necessary to a better understanding of the behaviour of organic anions in soils. This sort of integration has been achieved in studies combining experimental and modelling work (Geelhoed et al., 1999; Jones et al., 1996a) and has proved very useful in unravelling the real potential of these mechanisms involving organic anions in soil systems.

Aluminium effects on nutrient uptake and release of citrate by roots

The assessment of the spatial localisation of nutrient uptake on the root axis revealed that Al is affecting nutrient uptake widely along the longitudinal axis of the root. When this pattern of Al-induced inhibition of nutrient uptake was compared with the pattern of citrate exudation along identical root axes of the Al resistant genotype CMS36, assessed in Chapter 3, we noted that, interestingly, they do not match. Actually they were quite distinct from each other since the highest rates of citrate exudation were observed in root regions where uptake of calcium and magnesium were the lowest. Based on these findings it is, in principle, suggested that local citrate exudation is not directly involved in making plants more resistant to Al by maintaining nutrient uptake, but more by detoxifying Al around the root meristems, the most sensitive part for root growth. In plants grown in soil systems, however, the intermediate root segment, i.e. the zone with almost no citrate exudation, may benefit from the citrate exuded by the root apex and left in the soil. This citrate can probably protect the intermediate root zone against Al when following the apex through a citrate-enriched environment. Maintaining the uptake of nutri-

ents on a higher rate is probably a secondary and additional effect of root citrate exudation to make plants more resistant to Al.

Aluminium effects on the root citrate dynamics

Although the exudation of citrate by roots exposed to Al has been well characterised, the corresponding Al-induced changes in citrate production and in the citrate status of the root have not yet been comprehensively studied. It would be interesting to investigate firstly how Al is affecting the enzymatic machinery of root cells concerning the organic anion metabolism. Also, what changes in the synthesis of organic anions, and in the activities of key enzymes like citrate synthase, malate dehydrogenase, and phospho*enol*pyruvate carboxylase are necessary to meet the demand for root citrate exudation.

Secondly, aspects of altered organic anion metabolism and their dynamics within the plant induced by Al stress could be studied. For instance it was demonstrated with plants of oilseed rape suffering from P deficiency, that citrate produced in the shoot was transported via the phloem towards the root where it was accumulated in the root region responsible for exudation (Hoffland et al., 1992). Thus, the dynamics of citrate (e.g. production, consumption, transport, storage) at the plant level might be approached.

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Summary

Inhibition of root elongation, considered the primary or initial response of the plant to aluminium (Al) toxicity may have severe consequences to the overall plant performance, resulting finally in restricted shoot growth, reduced yield of crop plants, and lower quality of the final agricultural product (Rengel, 1992a). Considerable variation in resistance to Al exists among plant species and genotypes, and efforts to select and breed maize (*Zea mays* L.) germplasm with superior resistance to Al have been made worldwide. Determination of plant genetic, physiological, and biochemical mechanisms by which plants resist to Al stress is therefore an important part of the proposed plant breeding approach. Root exudation of small organic anions such as citrate, malate, and oxalate that can complex Al in the root apoplast and/or rhizosphere has been mentioned as a possible mechanism underlying Al resistance of certain plant species or cultivars. The general aim of this research was therefore to study the root exudation of these small ligands as a potential mechanism of Al resistance operating in maize.

Initially (Chapter 2) we studied genotypic variation for Al resistance in maize with a collection of ten maize genotypes obtained from the National Maize and Sorghum Research Centre (CNPMS), EMBRAPA, Brazil, using three different screening techniques. We aimed to rank the genotypes on Al resistance and to select the ones showing significantly different sensitivity to Al for further research on the root exudation of organic anions in response to Al. We also evaluated several plant characteristics that might on a short-term basis, indicate the general plant resistance to Al. Resistance to Al varied widely among the ten maize genotypes, as revealed by the different screening techniques used. The best indicators of differential Al resistance were root characteristics, especially root length. Internal root concentrations of citrate and malate, however, did not reflect plant resistance to Al. Two genotypes [CMS36 (Al resistant) and BR106 (Al sensitive)], representing the extremes of Al sensitivity within the collection that was available to us, were selected for further studies.

In a succeeding study (Chapter 3) qualitative and quantitative aspects of root exudation of organic anions in response to Al exposure were evaluated. Roots of maize seedlings were grown axenically in nutrient solution and root exudates were collected along the whole seminal root axis for short time periods (4 h) using a divided-root-chamber technique. In root exudates collected from 10-mm long root apices, citrate accounted for 67% of the total organic anions found, followed by malate (29%), *trans*-aconitate (3%), fumarate (<1%), and *cis*-aconitate (1%). Rates of citrate exudation from root apices of the two above-mentioned genotypes exposed to a range of external Al concentrations (0–

100 μM Al) were consistently higher in the Al resistant maize genotype CMS36. Furthermore, relative Al resistance of eight maize genotypes correlated significantly well with their citrate exudation rate measured at 40 μM Al. Higher exudation rates were accompanied by a less inhibited root elongation. The exudation of citrate along the longitudinal axis of fully developed seminal roots showed a particular pattern: citrate was exuded mainly in the regions of root apices, either belonging to the main root or to the lateral roots in the most basal part of the main root. Qualitatively we concluded that protection of the root by citrate seems more conceivable in the places of high exudation rates. In the root zones behind the apex, either citrate exudation rate is high enough to protect this less Al sensitive root region or this zone with almost no citrate exudation may benefit from the citrate exuded before by the root apex. This citrate can probably protect the intermediate root zone against Al when following the apex through a citrate-enriched soil environment. A more quantitative approach was made in Chapter 7 (see later in this Summary).

In Chapter 4 we sought the literature about the effects of Al on processes involved in the status of citrate in plant roots, specifically biosynthesis, transport, accumulation, and exudation. In addition, we tested experimentally some of the theories described in literature by studying the effects of external Al on the internal root citrate status and associated root citrate exudation rates along intact root axes of the Al resistant CMS36. External Al led to enhanced internal root citrate concentrations. Simultaneously, Al increased the citrate permeability of root cells, but this change in permeability seemed restricted to the root apices. Consequently, root apices of the Al resistant maize genotype showed enhanced rates of citrate exudation under conditions of Al stress. This exudation followed a particular pattern along the root axis with a well defined longitudinal distribution, and when compared to the corresponding distribution of internal citrate concentration, indicated that the concentration of citrate itself is not the driving force for citrate exudation from roots. It is suggested that dynamics of processes involved in citrate production, transport, and exudation operate differently along the longitudinal axis of the root and the pattern of citrate accumulation within Al-treated roots observed at the end of the Al treatment period reflected the net result of a combination of these processes.

The effects of Al on nutrient uptake were also considered in this research (Chapters 5 and 6). In a first experiment (Chapter 5) we studied the effects of Al on nutrient uptake of maize genotypes differing in Al resistance and checked whether differences in mineral nutrition under Al stress correspond with differences in resistance to Al in maize. Generally, Al had negative effects on the uptake of macro and micronutrients. The relative uptake [(uptake at 100 μM Al/uptake at 0 μM Al)×100] of the nutrients studied varied from 22% to 157%, indicating the existence of intraspecific variation for such feature in the presence of Al. Despite showing significant reductions in uptake of calcium (Ca) and

magnesium (Mg), the maize genotypes showed a rather variable sensitivity to the Al stress imposed, which was related to their general resistance to Al previously assessed using root length as indicator. Under conditions of Al stress, genotypes more resistant to Al maintained a relatively higher absorption of both Ca and Mg than those more sensitive to Al. It was suggested that the ability of a genotype to maintain a less disturbed nutrient uptake under Al stress can be an important component in resistance to Al. Subsequently short-term experiments were established (Chapter 6) to study the direct effects of Al on nutrient uptake, once in the previous study the direct, or primary, effects of Al on nutrient uptake could not be separated from the indirect, or secondary, effects on root and shoot growth, affecting plant's uptake capacity (root) and plant's nutrient requirement (shoot) due to the relatively long period of exposure to Al.

With CMS36 and BR106 uptake of Ca, Mg, and K was studied by complete seminal roots and along the whole axis of intact seminal roots of seedlings grown with or without Al. Exposure to Al reduced total uptake of Ca and Mg, but not that of K, which was actually stimulated by Al. The negative effects of Al on Ca and Mg uptake were more pronounced in the Al sensitive genotype. The assessment of the spatial localisation of nutrient uptake on the root axis revealed that Al is affecting nutrient uptake widely along the longitudinal axis of the root. When this pattern of Al-induced inhibition of nutrient uptake was compared with the pattern of citrate exudation along identical root axes of the Al resistant genotype CMS36, assessed in Chapter 3, we noted that, interestingly, they do not match. Actually they were quite distinct from each other since the highest rates of citrate exudation were observed in root regions where uptake of Ca and Mg were the lowest. We concluded that citrate is probably primarily involved in making plants resistant to Al by detoxifying Al around the root meristems, the most Al sensitive part for root growth. Local citrate exudation does not seem to be directly involved in nutrient uptake, because the segment with the highest citrate exudation (the apex) shows almost no nutrient uptake, while the root zone with the highest nutrient uptake shows almost zero citrate exudation. However, with plants grown in soil systems the intermediate root segment, i.e. the zone with almost no citrate exudation, may benefit from the citrate exuded before by the root apex. This citrate can probably protect the intermediate root zone against Al when following the apex through a citrate-enriched soil environment. This could explain the positive role of citrate, exuded by the apex, in nutrient uptake by 'older' root parts grown in acid soils. Maintaining the uptake of nutrients on a higher rate, is probably a secondary and additional effect of root citrate exudation to make plants more resistant to Al.

In Chapter 7 a quantitative analysis on the role of organic anions in the mechanism of Al resistance was done. The question whether quantitatively the amounts of these small ligands released in the root environment are adequate to explain resistance to Al was

tackled by combining experimental and modelling work. We hypothesised that resistance to Al is achieved via modification of the apoplastic environment to effectively protect cell wall and cell membrane function from toxic Al. We used a mechanistic model to describe the dynamic build-up of citrate concentrations in the root apoplast and citrate diffusion to the outer root medium, and confronted the final outcome of the calculations with observed experimental data. The potential of amounts of exuded citrate to complex Al under local conditions was predicted with a detailed chemical speciation model. The model calculations suggest that Al activity in the apoplastic compartment can only be kept lower than in the (soil) solution in a dynamic system, where a continuous efflux of a complex forming agent may exist. The results strongly support the notion that citrate indeed can underlie Al resistance in maize. For the conditions considered in this study, detoxification of apoplastic Al and protection of this compartment seem more realistic and more important than those in the interface root-outer solution. A careful extrapolation of how the processes might work in soil systems indicated an even higher potential of this mechanism of protection of roots against Al in soils. Important factors that might well strengthen the role of organic anions are discussed.

Samenvatting

Remming van de toename in wortellengte, veelal beschouwd als de primaire of initiële respons van de plant op aluminium (Al) vergiftiging kan ernstige gevolgen hebben voor het algeheel functioneren van de plant en uiteindelijk resulteren in beperkte spruitontwikkeling, verminderde opbrengst bij cultuurgewassen, en een afname van de kwaliteit van het eindproduct bij landbouwgewassen (Rengel, 1992a). Er bestaat een aanzienlijke variatie in tolerantie tegen Al tussen plantensoorten en tussen rassen en over de hele wereld worden door veredelaars pogingen ondernomen om maïs 'germplasm' te selecteren met een verhoogde tolerantie tegen Al. Het vinden van genetische, fysiologische en biochemische mechanismen die de plant meer tolerant maken tegen een overmaat aan Al is daarnaast een belangrijke onderdeel van het veredelingsonderzoek. Uitscheiding door de wortel van kleine organische anionen, zoals citraat, malaat en oxalaat, die Al kunnen complexeren in de wortel apoplast en/of rhizosfeer zijn genoemd als een mogelijk mechanisme dat ten grondslag ligt aan de Al tolerantie van bepaalde plantensoorten of rassen. Het algemeen doel van dit onderzoek was daarom om de uitscheiding van dergelijke kleine liganden door de wortel te bestuderen als een mogelijke mechanisme van Al tolerantie bij maïs (*Zea mays* L.).

Allereerst (Hoofdstuk 2) bestudeerden we door middel van een drietal selectietechnieken verschillen in Al tolerantie tussen maïs genotypen bij een verzameling van tien maïsrassen afkomstig van het Nationaal Maïs en Sorghum Onderzoek Centrum (CNPMS), EMBRAPA, Brazilië. Het doel van dit onderzoek was tweeledig. Allereerst werden de rassen onderling gerangschikt op basis van hun tolerantie tegen Al en vervolgens werden de rassen die onderling significant verschilden in hun Al gevoeligheid geselecteerd voor vervolgonderzoek naar hun vermogen om organische anionen uit scheiden na blootstelling aan Al. We onderzochten eveneens diverse karakteristieken van de plant die bij kortdurende blootstelling aan Al geschikte indicatoren voor Al tolerantie zouden kunnen zijn. De drie gebruikte selectietechnieken toonden aan dat tolerantie tegen Al sterk verschilde tussen de tien rassen. De beste indicatoren voor verschillen in Al tolerantie waren wortel karakteristieken, in het bijzonder wortellengte. Gehalten aan citraat en malaat in de wortel bleken niet de mate van Al tolerantie van de plant te weerspiegelen. De rassen CMS36 (Al tolerant) en BR106 (Al gevoelig) waren de twee meest extreme rassen binnen de verzameling van tien onderzochte rassen. Deze twee rassen werden geselecteerd voor verdere studies.

In de volgende studie (Hoofstuk 3) werden kwalitatieve en kwantitatieve aspecten van de uitscheiding van organische anionen als gevolg van blootstelling aan Al bes-

tudeerd. Wortels van maïs zaailingen werden allereerst in een voedingsoplossing steriel gekweekt. Vervolgens werd gedurende 4 uur door middel van een zogenaamde 'gescheiden-wortelkamer techniek' de uitscheiding van organische anionen langs de gehele, intacte hoofdwortel gemeten. Dit gebeurde met deze techniek per 10-mm wortelsegment, vanaf de wortelpunt tot het meest basale (oudste) deel van de wortel. Van alle organische anionen uitgescheiden door het jongste 10-mm wortelsegment bestond 67% uit citraat, gevolgd door malaat (29%), trans-aconitaat (3%), fumaraat (<1%), en cis-aconitaat (1%). De snelheid waarmee citraat werd uitgescheiden door het jongste wortelsegment was bij het Al tolerante ras CMS36 aanzienlijk hoger dan bij BR106. Dit gold voor een breed traject aan externe Al concentraties in het wortelmedium (0–100 μM Al). Verder bleek de mate van Al tolerantie van de maïsrassen significant gecorreleerd te zijn met de snelheid van citraatuitscheiding bij een Al concentratie van 40 μM. Hogere snelheden van citraat uitscheidingen gingen samen met een minder sterke remming van de wortelontwikkeling. De verdeling van de citraatuitscheiding door de wortel, gemeten langs de longitudinale as van de volledig ontwikkelde hoofdwortel, vertoonde een opvallend patroon; citraat werd voornamelijk uitgescheiden door de worteltoppen, óf behorend tot de hoofdwortel, óf tot de laterale wortels voorkomend in de meest basale delen van de wortel. Op kwalitatieve gronde werd door ons geconcludeerd dat bescherming van de wortel door citraat meer aannemelijk lijkt voor delen van de wortel met een hoge snelheid aan citraatuitscheiding, de worteltoppen. In de wortelzones achter de top is óf de citraatuitscheiding voldoende om deze minder Al-gevoelige delen van de wortel tegen Al toxiciteit te beschermen, óf deze wortelzones met nagenoeg geen citraatuitscheiding kunnen profiteren van citraat eerder uitgescheiden door de worteltop. Dit citraat kan wellicht die delen van de wortel beschermen doordat ze de worteltop volgen door een met citraat verrijkt bodemcompartiment. Een meer kwantitatieve benadering omtrent de potentie van het uitgescheiden citraat om Al te kunnen detoxificeren volgt in Hoofdstuk 7 (zie ook later in deze Samenvatting).

In Hoofdstuk 4 werd een literatuurstudie beschreven naar de effecten die Al heeft op het voorkomen van citraat in plantenwortels, in het bijzonder productie, transport, ophoping en uitscheiding. Daarnaast werden enkele theorieën experimenteel getoetst. Zo werd bij het Al-tolerante ras CMS36 het effect van de aanwezigheid van Al in het wortelmedium getoetst op het verloop van het gehalte aan citraat in de wortel langs de intacte hoofdwortel in samenhang met de locale uitscheiding van dit organisch anion door de diverse wortelregionen. De aanwezigheid van Al leidde tot verhoogde gehalten aan citraat in de wortel. Tegelijkertijd verhoogde Al de permeabiliteit van wortelcellen voor citraat, maar deze verandering in permeabiliteit leek zich te beperken tot de worteltoppen. Als gevolg hiervan vertoonden de worteltoppen van het Al-tolerante ras verhoogde uitscheiding van citraat na blootstelling aan Al. De citaatuitscheiding zoals

die werd gemeten langs de intacte hoofdwortel vertoonde een bijzonder en vast patroon. Een vergelijk met de verdeling van het citraat in de wortel toonde aan dat de citraat concentratie in de wortel zeker niet de drijvende kracht kan zijn voor de snelheid waarmee citraat wordt uitgescheiden door de wortel. Aangenomen wordt dat de dynamiek van processen die betrokken zijn bij productie, transport en uitscheiding van citraat varieert langs de longitudinale as van de hoofdwortel. De verdeling van de citraatophoping binnen de wortels van aan Al blootgestelde planten weerspiegelt uiteindelijk het netto resultaat van een combinatie van eerdergenoemde processen.

De effecten van Al op de nutriëntenopname werden eveneens bestudeerd in dit onderzoek (Hoofdstukken 5 en 6). In een eerste onderzoek (Hoofdstuk 5) werden de effecten van Al bestudeerd op de nutriëntenopname van maïsrassen welke verschilden in Al tolerantie en werd getoetst of verschillen in minerale voeding onder omstandigheden van Al stress correspondeerden met verschillen in tolerantie tegen Al bij maïs. In het algemeen had Al negatieve effecten op de opname van zowel macro- als micronutriënten. De relatieve opname [(opname bij 100 μM Al/opname bij 0 μM Al)×100] van de nutriënten die bestudeerd werden varieerde van 22% tot 157%, hetgeen wijst op de specifieke rol van Al op de opname van afzonderlijke nutriënten. Ondanks de significante afname in opname van calcium (Ca) en magnesium (Mg) vertoonden de maïsrassen een nogal grote variatie in gevoeligheid voor Al, gebaseerd op hun verschil in wortelontwikkeling onder omstandigheden van Al stress, zoals eerder gemeten (Hoofdstuk 2). Bij blootstelling aan Al handhaafden de meer Al-tolerante rassen een hogere relatieve opname aan zowel Ca en Mg dan de gevoeligere rassen. Er werd dan ook gesuggereerd dat het vermogen van een ras om onder omstandigheden van Al stress de afname in nutriëntenopname te kunnen beperken een belangrijk onderdeel kan zijn van tolerantie tegen Al.

Vervolgens werden kortdurende experimenten uitgevoerd (Hoofdstuk 6) om de directe invloed van Al op de nutriëntenopname te bestuderen. Dit werd gedaan omdat in de voorafgaande studie de directe of primaire effecten van Al op de nutriëntenopname niet ontkoppeld konden worden van indirecte of secondaire effecten, effecten op wortel- en spruitgroei die op hun beurt de opnamecapaciteit (wortel) en behoefte aan nutriënten (spruit) beïnvloeden als gevolg van de relatief lange blootstellingduur aan Al.

Met CMS36 en BR106 werd de opname aan Ca, Mg en K bestudeerd bij complete hoofdwortels evenals het opnamepatroon langs de gehele as van intacte hoofdwortels van zaailingen gekweekt in de aan- en afwezigheid van Al. Blootstelling aan Al verminderde de totale opname aan Ca en Mg, maar niet die aan K, die zelfs toenam na Al blootstelling. De negatieve effecten van Al op de opname van Ca en Mg waren sterker in het Al gevoelige dan in de Al-tolerante ras. Bepaling van de ruimtelijke verdeling van de nutriëntenopname langs de hoofdwortel toonde aan dat Al de nutriëntenopname over een breed traject van de wortelas beïnvloedt. Een vergelijk van het patroon van remming in

nutriëntenopname als gevolg van Al toediening met het patroon van citraatuitscheiding, beiden gemeten langs de verticale as van de hoofdwortel van het Al-tolerante ras CMS36, vermeld in Hoofdstuk 3, leverde de interessante constatering op dat beide patronen niet samenvallen. In werkelijkheid verschilden ze onderling nogal omdat de hoogste snelheden aan citraatuitscheidingen werden waargenomen in die wortelregionen waar de opname aan Ca en Mg juist het laagst was. Door ons werd dan ook geconcludeerd dat de primaire rol van citraat in Al tolerantie mechanismen bij planten werkt via het ontgiftigen van Al rond de wortelmeristemen, het meest Al-gevoelige deel van de wortel wat groei betreft. Locale uitscheiding van citraat schijnt niet direct betrokken te zijn bij de nutriëntenopname, omdat het wortelsegment met de hoogste citraatuitscheiding (de top) nagenoeg geen opname te zien geeft, terwijl bij de wortelzone met de hoogste nutriëntenopname de citraatuitscheiding nagenoeg ontbreekt. Echter, met planten geteeld op grond kan wellicht het intermediaire worteldeel, i.e. de wortelzone met nagenoeg geen citraatuitscheiding, profiteren van het citraat dat eerder door het wortelpunt is uitgescheiden. Dit citraat kan mogelijk het intermediaire deel van de wortel beschermen, wanneer het de worteltop volgt door grond die eerder met citraat verrijkt is. Dit zou de positieve rol van citraat, uitgescheiden door het wortelpunt, kunnen verklaren met betrekking de nutriëntenopname door ‘oudere’ worteldelen groeiend in zure gronden. Handhaving van de nutriëntenopname op een hoger niveau is waarschijnlijk een secondair en additioneel effect van citraatuitscheiding om planten meer tolerant te maken tegen Al.

In Hoofdstuk 7 is een kwantitatieve analyse van de betekenis van organische anionen in het mechanisme van Al tolerantie gepresenteerd. De vraag of de hoeveelheden van deze kleine liganden die in de wortelomgeving worden uitgescheiden kwantitatief voldoende zijn om de tolerantie tegen Al te verklaren werd aangepakt door experimenteel werk te combineren met modellering. Uitgangspunt hierbij was de hypothese dat tolerantie tegen Al is verkregen via modificatie van het apoplasma door effectief de functies van celwanden en celmembranen te beschermen tegen toxisch Al. We gebruikten daarbij een mechanistisch model om de dynamische opbouw van citraat concentraties in het wortelapoplasma en de citraat diffusie naar het buitenmedium van de wortel (rhizosfeer) te beschrijven. Vervolgens werden de uitkomsten van de berekeningen vergeleken met waarnemingen uit experimenten. Het vermogen van de hoeveelheid door de plant uitgescheiden citraat om Al onder de locale omstandigheden te complexeren is voorspeld met een gedetailleerd chemisch speciatiemodel. De modelberekeningen geven aan dat de Al activiteit in het apoplasma alleen lager kan zijn dan in bodem- of voedingoplossing wanneer sprake is van een dynamisch systeem met en continue efflux van een complexvormende agens. De resultaten ondersteunen sterk het idee dat citraat inderdaad ten grondslag kan liggen aan de tolerantie tegen Al bij maïs. Voor de condities waarvan is

uitgegaan in deze studie schijnen ontgiftiging van Al in het apoplasma en bescherming van dit cel compartiment realistischer en belangrijker dan effecten op het grensvlak wortel-buitenmedium. Een voorzichtige extrapolatie hoe de processen zouden kunnen werken in bodem systemen duiden zelfs op een betere werking van dit mechanisme om wortels te beschermen tegen Al in zure bodems. Belangrijke factoren die de rol van organische anionen zouden kunnen versterken worden bediscussieerd.

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About the author

Eduardo Dal'Ava Mariano was born on April 5, 1973 in Poços de Caldas, MG, Brazil. He studied Agronomy at the Federal University of Lavras (UFLA), Lavras, MG, Brazil from March 1991 to December 1995. Subsequently he followed an MSc programme in Soil Science and Plant Nutrition at the same university, obtaining his MSc degree in August 1998. In January 1999 he started his PhD studies at Wageningen University, Wageningen, The Netherlands, on root exudation of organic anions as a mechanism of aluminium resistance in maize. He completed his PhD studies in June 2003.