Al toxicity and plant nutrient uptake: a role for root cell walls, pH and organic chelators

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Al toxicity and plant nutrient uptake: a role for root cell walls, pH and organic chelators

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I rörelse

Den mätta dagen, den är aldrig störst.
Den bästa dagen är en dag av törst.

Nog finns det mål och mening i vår färd -
men det är vägen, som är mödan värd.

Det bästa målet är en nattlång rast,
där elden tänds och brödet bryts i hast.

På ställen, där man sover blott en gång,
blir sömnen trygg och drömmen full av sång.

Bryt upp, bryt upp! Den nya dagen gryr.
Oändligt är vårt stora äventyr.

(Karin Boye, uit: ”Härdarna”, 1927)

aan mijn ouders

Abstract

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Soil acidification can lead to dissolution of aluminium (Al), which, in toxic concentrations, decreases growth and inhibits nutrient uptake in plants. The extent of Al toxicity depends on the ambient pH and on the presence of organic chelators, like citrate. In this thesis, we focus on the Al interference with plant uptake of macro- and micronutrients under different environmental conditions.

Al accumulates in the root apoplast of most plants. There it adsorbs to negative sites on the cell walls, which under normal circumstances are occupied by Ca and other nutrient cations. Adsorption to the cell wall possibly assists in nutrient uptake into the cell. We showed the capacity of Al to replace Ca, Mg, Zn, Cu and Mn at cell wall binding sites, in competition experiments with isolated cell wall material from tomato roots. Both a low pH and the presence of the metal chelator citrate and EDTA strongly decreased Al adsorption. The competition between Al, Ca and H for cell wall binding was described with a Gaines-Thomas exchange model.

In plant experiments with tomato and wheat, representing di- and monocots with their difference in cation binding capacity of the cell wall, Al exposure led to a large decrease in uptake of the aforementioned nutrients. Especially Cu and Zn uptake was inhibited, causing even a net efflux in wheat roots. A lower pH in the root apoplast decreased Al accumulation and could prevent the Al effects on Cu and Zn uptake. Complexation of Al by citrate or EDTA generally improved plant growth and its nutrient concentrations but interfered with Cu and Zn uptake. In the case of citrate, this could mean a negative side effect of organic anion exudation as a mechanism for Al tolerance in plants.

Key words: Al phytotoxicity, plant nutrient uptake, cell wall adsorption, organic chelators, citrate, calcium, magnesium, copper, zinc, pH.

Content

List of abbreviations

$[Al]_{rt}$	Aluminium concentration in the root (mol (g root DM)$^{-1}$)
$\{cation^{a+}\}_{sol}$	activity of cation with valency a in the (adsorption) solution (mol L^{-1})
$[cation]_{NS}$	cation concentration in the nutrient solution (mol L^{-1})
$[cation]_{sol}$	cation concentration in the solution (mol L^{-1})
$[cation]_{cw}$	cation adsorption to the cell wall (mol or eq (g DCW)$^{-1}$)
CEC	cation exchange capacity (eq (g DCW)$^{-1}$)
cit	citrate
DCW	dry cell wall
DM	dry matter
ECOSAT	Equilibrium Calculation Of Speciation And Transport
EDTA	(Na_2-) ethylenediamine tetraacetic acid
E_{cat}	equivalent fractions of bound cations (-)
FiNC	Fictitious Nutrient Concentration (mol g^{-1})
ICP-AES	inductively coupled plasma atomic emission spectrometry
ICP-MS	ICP- mass spectrometry
$K^{GT}_{Ca\backslash H}$	Gaines-Thomas exchange coefficient; subscript indicates that H is exchanged for Ca
LMW	low molecular weight (organic acids)
mal	malate
Mha	mega hectare
NS	nutrient solution
Q_{max}	total number of adsorption sites (eq (g DCW)$^{-1}$)
RGR	relative growth rate (day^{-1})
RL	root length (m)
RUR	relative uptake rate (day^{-1})
SRL	specific root length (m g^{-1})

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General Introduction

Al in the environment: an acid soil problem

Aluminium is one of the most abundant metals in the soil and accounts for 7-8% of the earth crust (Lindsay and Walthall, 1989; RSC, 2003). In its mineral form Al poses no threat to plants but increased acidification and weathering of a soil dissolves Al-containing minerals and desorbs Al from the surface of soil particles, thus releasing phytotoxic Al^{3+}. A combination of factors will determine how toxic the Al in solution will be for a plant: e.g. soil pH and activities of other cations than Al^{3+} and H^{+}, soil organic matter content and the presence of mycorrhiza. A low pH will not only exchange Al at the surface sites of soil particles but also nutrient cations will be replaced, causing leaching of Al and base nutrient cations to deeper soil layers (Gustafsson *et al.*, 2001; Lundin *et al.*, 2001; Zelazny and Jardine, 1989). The pH in the soil will also determine the chemical speciation of Al (Göttlein *et al.*, 1999; van Hees *et al.*, 2001). At a pH $\leq$ 4, most Al will be present as Al^{3+}, while above pH 4, increasing concentrations of Al-hydroxides will be formed. The presence of high concentrations of base cations can prevent Al toxic effects by decreasing its activity in the solution and by competing with Al for plant uptake. A ratio of (Ca+Mg)/Al, often also including K in the numerator, is used as the chemical criterion in evaluating the critical load of acid deposition in a soil. These days the validity of the ratio is under discussion, since it does not distinguish between high and low Al concentrations and it disregards biological factors in acid soil toxicity, like vegetation type or mycorrhiza (Göransson and Eldhuset, 2001; Løkke *et al.*, 1996). A high organic matter content in the soil will decrease Al toxicity by adsorbing free Al^{3+} (Haynes and Mokolobate, 2001; Hue *et al.*, 1986; Ritchie, 1995). Plants on organic soils with a very low pH will therefore usually suffer more from proton toxicity than from Al, while in acid mineral soils Al toxicity will prevail (Kidd and Proctor, 2001; Kinraide, 2003). The fact that Al bound to soil organic matter decreases organic matter dissolution and decomposition shows that even adsorbed Al can have an impact on the ecosystem (Mulder *et al.*, 2001). A symbiosis between a plant root and certain mycorrhizal fungi can decrease toxic effects of Al for a plant by interception and adsorption of (part of the) Al before it reaches the root or by exudation

of Al-chelators (Cuenca *et al.*, 2001; Jentschke and Godbold, 2000; Joner *et al.*, 2000; Keltjens, 1997).

Acid soils occupy approximately 30% or 3950 Mha of the world's ice-free land area, 67% of which is covered by forests and woodlands (Von Uexkuell and Mutert, 1995). Agricultural use of acid soils is often problematic because of the low pH, high contents of soluble Al and a low base cation content. Constant application of lime and fertiliser would solve many of the problems but this is not always a feasible option in developing countries (FAO, 2000). The main source of soil acidification over the past century has been the increasing acid atmospheric deposition related to human activity. Important sources of pollution are agriculture, industry and traffic, releasing large amounts of ammonia and nitrous and sulphuric oxides, which form acids in the soil (Bleeker and Erisman, 1996; Hovmand, 1999; Van Breemen *et al.*, 1982). Acid concentrations can even be higher close to e.g. a tree as a result of stemflow, when depositions on the plant are rinsed off (Matschonat and Falkengren-Grerup, 2000). Atmospheric depositions cannot be confined to the country of origin and once its impact became known, many countries took action to reduce emission and international co-operations were set up to monitor ecosystems for harmful effects (Gregor *et al.*, 2001; Lundin *et al.*, 2001). Long term acid deposition has been linked to a decrease in forest growth (Hovmand and Bille-Hansen, 1999; Ouimet *et al.*, 2001). However, some studies indicate that the effect on the vegetation is caused by more than soil acidification alone and that effects may vary with rooting depth (Carnol *et al.*, 1999; Derome *et al.*, 2001).

Al uptake into the plant

Al enters the plant root with the uptake of soil solution and accumulates in most plant species in the epidermal and cortex cell layers of the root apoplast. The accumulation is a consequence of different factors: firstly, the positively charged Al^{3+} strongly interacts with negatively charged binding sites in the root cell walls and the outer membrane surface. These sites are under Al-free conditions occupied by Ca and other nutrient cations (Keltjens, 1995; Kuhn *et al.*, 1995). Secondly, transport of Al over the plasma membrane into the cell is very limited (Rengel and Reid, 1997; Taylor *et al.*, 2000).

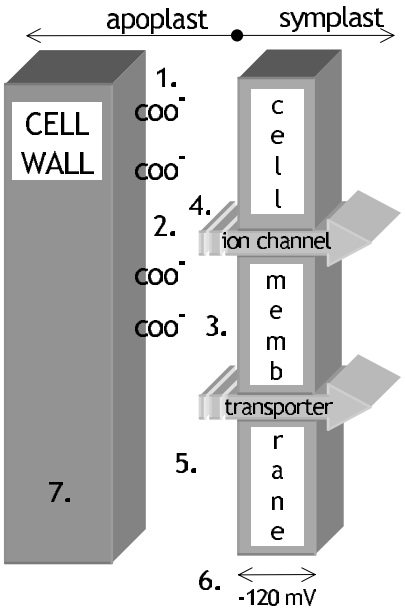
Mass flow will transport it through the root apoplast, which is the plant compartment outside the plasmalemma, consisting of cell walls, cell wall pores and intracellular spaces (Sattelmacher, 2001). At the Casparian strip of the endodermis, the water flow enters the plant symplast when crossing the plasma membrane, leaving Al behind in the cortex (Göransson and Eldhuset, 1995; Marienfeld *et al.*, 2000). Al can enter the cell at a very low rate but an uptake mechanism for Al into the cell has so far not been identified. Most plant species will thus contain the Al in their root system, yet some species, like tea (*Camellia sinensis*) and buckwheat (*Fagopyrum esculentum*) can transport and accumulate Al in high concentrations in the shoot (Ma and Hiradate, 2000; Nagata *et al.*, 1993).

Al phytotoxicity

Al, in toxic concentrations, will reduce plant growth and nutrient uptake (Delhaize and Ryan, 1995; Kidd and Proctor, 2000; Rout *et al.*, 2001). The first symptom of Al toxicity is usually a reduction in root growth as a result of inhibited cell elongation and it is this feature, which is most often used as an indicator for Al toxicity. The root apex, where Al accumulates the most, is especially sensitive (Horst, 1995; Ryan and Kochian, 1993). Some of the possible targets for Al interference in the root apoplast are depicted in the figure on the next page. Al can replace cations on the carboxylic binding sites of the root cell wall (1; Schmohl and Horst, 2000). Ca is the major cation adsorbed to cell walls, creating cross-links between pectic chains, and Al interferes by replacing Ca. Other cations, like Mg and Cu, also adsorb to the cell wall and may be replaced by Al. On the cell wall, Al may interfere with enzyme functioning in cell wall metabolism (Horst, 1995). Binding to cell wall pectins may interfere with intracellular processes through disturbance of Ca-homeostasis and of the cell wall-plasma membrane-cytoskeleton continuum (Horst *et al.*, 1999; Rengel,1992). The replacement of divalent cations by the trivalent Al^{3+} may cause a difference in charge division at the cell wall surface, affecting total cation adsorption (2). Similarly, Al replaces cations on the plasma membrane surface, changing its functions and surface charge (3; Matsumoto, 2000). Al can compete with nutrient cations for membrane transporters and ion channels and subsequent uptake into the cell (4).

It interferes directly with Ca and K uptake by inhibiting their specific cation channels (Ding *et al.*, 1993; Liu and Luan, 2001).

Disturbing Al causes a change in apoplast pH by inhibiting plasma membrane H^+-ATPases, which maintain a proton efflux from the cell (5; Ahn *et al.*, 2002). Because of this change in apoplast pH, the reduced proton efflux and an Al-induced inhibition of K^+-selective ion channels the membrane electrical potential, which drives cation uptake, decreases (6).



1. *Cation replacement on the cell wall carboxylic binding sites; change in cross-linking of cell wall pectin; interference with cell wall enzymes.*
2. *Change in cell wall charge division.*
3. *Change in plasma membrane functions and surface charge.*
4. *Competition with nutrient cations for uptake into the cell.*
5. *Change in apoplast pH as a result of interference with e.g. membrane proton fluxes.*
6. *Decrease of membrane electrical potential.*
7. *Decrease of apoplast volume.*

Figure: *Possible Al effects in the root apoplast*

Finally, Blamey and Dowling (1995) reported a decrease in volume in a Ca-pectate matrix as a result of Al exposure. If this is a consequence of a change in pectin cross-linking, like mentioned under (1), a similar mechanism may lead to a tighter cell wall matrix with reduced pore size. This in turn could possibly lead to decreased flow through the apoplast and a subsequent inhibition of nutrient uptake (7). This list can most likely be extended with other known and unknown effects of Al interference in the root apoplast.

Al and pH will affect each other in the plant root: the pH will determine Al speciation, like it was mentioned in the first paragraph of this chapter, and Al changes proton fluxes. Both factors have to some extent similar toxic effects on plant growth and nutrient uptake (Koyama *et al.*, 2001; Smith and Krikorian, 1992). Together they may work in concert to aggravate effects on the plant, yet at low concentrations they can mutually alleviate each other's toxicity (Kidd and Proctor, 2001; Kinraide, 1993).

Al tolerance and -sensitivity in plants

Different plants species or even cultivars respond very differently to Al in the solution. An Al-sensitive plant species may show severe symptoms of Al toxicity at a given concentration, whereas a more tolerant species appears completely unaffected. Over the years, a range of plant mechanisms, which allow a plant to tolerate Al in the rhizosphere or in its tissues, have been identified and studied. Initially a high capacity of the cell wall to bind and store Al was considered an advantage for the plant, though this view has since been abandoned because of lack of correlation with Al tolerance (Horst, 1995). More relevant was the discovery that many Al-tolerant plants can exude organic anions, which bind and detoxify Al. An important group among these chelators is formed by the low molecular weight (LMW) organic anions, like citrate, oxalate and malate. Contact of a root, possibly more specifically the root border cells, with Al can induce the release of an internal organic anion storage and synthesis of new organic acids (Kollmeier *et al.*, 2001; Miyasaka and Hawes, 2001). External (rhizosphere) or apoplastic complexation of Al prevents it from interacting with the root apoplast and Al accumulation is reduced. Some plants, like the Al accumulator tea, can take up Al-organic anion complexes and transport it to the shoot, storing it as a non-toxic form of Al within the plant (Ma *et al.*, 1997b; Ma *et al.*, 1998). The mycorrhizal fungi mentioned in the first paragraph of this chapter can exude organic anions and bind Al and may also increase organic acid exudation in the plant root (Ahonen-Jonnarth *et al.*, 2000; Jentschke and Godbold, 2000). LMW organic anions are not the only plant produced Al-chelators: plants can exude phenolic compounds which can be very effective in binding Al (Barceló and Poschenrieder, 2002; Kidd *et al.*, 2001).

Other Al tolerance mechanisms include a root induced increase in rhizosphere pH, which causes Al to precipitate, and root proliferation in areas where Al concentration is lower (Degenhardt *et al.*, 1998; Hairiah *et al.*, 1992; Keltjens, 1997). Understanding Al-tolerance in plants can be a very useful tool in improving plant growth on acid soils. Together with pH-sensitivity it can be an explanatory factor for biodiversity of an ecosystem by determining the distribution of more or less Al-tolerant species (Brunet *et al.*, 1996; Schöttelndreier *et al.*, 2001). The interaction of aluminium, organic anions and pH forms the continuous thread in this thesis.

Aim of the research and outline of this thesis

Do organo-Al-complexes in the rhizosphere or in the root apoplast interfere in any way with plant growth and nutrient uptake or are they fully inert in that respect? What is the fate of these organo-Al complexes in the root; do they interact with the root apoplast and particularly with the cell wall? Which role can Al adsorption to the cell wall play in inhibition of nutrient uptake? How efficient and absolute is the counter-acting of Al toxicity by organic anions and what are the prerequisites for such an Al-detoxification? These are some of the questions that led to the start of this research project in 1996. Some questions were answered and more were raised.

The root apoplast, with its matrix of cell wall polymers, is the first compartment an Al ion encounters upon entering the root. In chapter 2 and 3, the interaction of Al with root cell walls and the competition of Al with other cations for cell wall binding are studied by performing *in vitro* adsorption experiments on isolated root cell walls from tomato. In chapter 2, the competition between Al^{3+}, Ca^{2+} and H^{+} for adsorption to the cell wall is described with a Gaines-Thomas exchange model. This competition is also studied in the presence of organic anions, to see if it changes Al interaction with the cell wall. Chapter 3 approaches the Al adsorption competition from a more biological point of view by taking a full nutrient solution as background. and describing the competition for cell wall binding between Al and nutrient cations in the solution, micronutrients inclusive. Citrate is used here as an Al chelator, like in chapter 2, as well as EDTA as a less easily degradable chelator in solution.

The following three chapters describe the effects of Al and Al-organic anion complexes in whole plant experiments. Tomato and wheat seedlings are chosen for these experiments as representatives of the di- and monocotyledons, respectively, which differ in cell wall binding capacity. Al accumulation, plant growth and nutrient uptake responses to Al(-EDTA) exposure are monitored over a period of several weeks (chapter 4). Chapter 5 describes the effect of ambient pH on Al toxicity, growth and nutrient uptake and chelator (EDTA) function. A second experiment presents a plant-induced change in pH and its effect on Al toxicity. The Al toxicity study in plants concludes with wheat exposure to Al(-citrate) with an adapted nutrient film technique, to prevent breakdown of the citrate in solution (chapter 6). An epilogue (chapter 7) gives a résumé of the main results of the previous chapters and places them in a broader perspective.

2

Aluminium-calcium-proton competition for *in vitro* adsorption to isolated tomato (*Lycopersicon esculentum* L.) root cell walls

Abstract

The *in vitro* adsorption of Al^{3+} and Ca^{2+} to isolated cell wall material of tomato (*Lycopersicon esculentum* L.) root was examined under different pH conditions and in the presence of an aluminium chelator like citrate and malate. Al displaced Ca from the binding sites to a large extent, with Al adsorption depending strongly on the pH of the solution. At extremely low pH, only little Al adsorbed on the cell wall material, supporting the view that inhibition of root elongation in acid soils involves more than Al^{3+} replacing Ca^{2+} at pectic cross-links. Al adsorption sites included both the carboxylic groups of the cell wall pectin and non-Ca binding groups, presumably cell wall proteins. Complexation of aluminium by organic anions decreased Al^{3+} competition with Ca^{2+} for adsorption sites. This effect was strongest at a moderately low pH, since at very low pH, organic acids will be protonated and do not bind Al^{3+} strongly. The competition of (organo-) Al^{3+}, Ca^{2+} and H^{+} for cell wall binding sites is described in a Gaines-Thomas exchange model and possible biological implications of the competition are discussed.

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Submitted

Introduction

A major restraint for plants growing on acid mineral soils is the presence of high concentrations of monomeric aluminium (Al^{3+}, Al-hydroxide) species. Root cell elongation is severely inhibited in Al-affected plants, an effect which is most pronounced in root tips and lateral roots (Silva *et al.*, 2001). Because the thus formed roots are short and thick, they cannot fully explore the soil and uptake of water and nutrients is reduced. The effect of aluminium on cell elongation can be overcome by addition of supplemental calcium or magnesium (Grauer and Horst, 1992; Keltjens and Tan, 1993; Silva *et al.*, 2001c). Additionally, many plant species can withstand phytotoxic Al concentrations by producing organic anions, like citrate, oxalate and malate to form complexes with the toxic metal; the control and the effectiveness of this exudation is still under investigation (Horst, 1995; Jorge and Arruda, 1997; Keltjens, 1997). Although aluminium phytotoxicity has been a focal point of scientific study for decades, its exact mechanism is still not known.

Aluminium affects the plant root cell wall composition, plasma membrane functioning, Ca homeostasis and cytoplasmic processes (Lindberg and Strid, 1997; Matsumoto *et al.*, 1996; Ryan *et al.*, 1997; Tabuchi and Matsumoto, 2001). Phytotoxic aluminium concentrations in the rhizosphere lead to decreased Ca levels in the plant and eventually to Ca deficiency but Al toxicity symptoms appear long before plant Ca concentrations decrease to below deficiency threshold levels. A disturbance in Ca^{2+} circulation within the apoplast and influx of Ca^{2+} into the cytosol, both of which are required for secretion of new cell wall material, can lead to inhibition of cell extension, loss of plasma membrane integrity and disturbance of Ca^{2+}-sensitive cation channels (Rengel, 1992; Zhang and Rengel, 1999).

Aluminium cannot easily be transported over the plant cell membrane and, upon entering the root, it accumulates in the apoplast of the root cortex (Horst, 1995; Rengel, 1992). In the root apoplast, the positively charged aluminium ion (Al^{3+}) interacts with carboxylic groups of the pectic matrix of the cell wall (Ma *et al.*, 1999; Schmohl and Horst, 2000; Zhang and Taylor, 1990), with proteins in the cell wall (Kenjebaeva *et al.*, 2001) and

with phospholipids and proteins situated on the outer surface of the cell membrane (Horst *et al.*, 1999; Kinraide *et al.*, 1992; Ryan *et al.*, 1997).

In the absence of aluminium and at neutral to slightly acidic pH these negatively charged binding sites are occupied mainly by protons or by calcium, which can be present in the root apoplast in mM concentrations (Rengel, 1992). A major fraction of Ca^{2+}ions in the apoplast is formed by the cross-links between polygalacturonic acids of the cell wall pectic chains (Carpita and Gibeaut, 1993; Horst *et al.*, 1999; Ryan *et al.*, 1997). Baydoun and Brett (1984) found over 60% of apoplastic Ca^{2+} to be associated in these cross-links in etiolated pea epicotyls. In growing cells the calcium cross-links are temporarily removed by apoplast acidification (as a result of proton extrusion from the cells) to allow cell elongation (Kutschera, 1994; McQueen-Mason, 1995; Yu and Tang, 2000). A higher affinity of Al^{3+} for the carboxylic groups of the pectic components would cause an increased rigidity of the cell wall since these cross-links would be less easily severed by proton extrusion in growing cells (Schofield *et al.*, 1998). Studies with tobacco spheroplasts suggest that Al^{3+} binds preferentially to newly formed pectin in growing cells (Chang *et al.*, 1999), which is in agreement with the strong Al toxic effect in elongating cells. Al-pectin cross-linking is also considered to reduce the pore volume of the apoplast, thus influencing the water flow and nutrient transport through the root (Blamey and Dowling, 1995; Horst *et al.*, 1999).

The pH in the root apoplast will influence the speciation and activity of the aluminium present. At low pH (< 4) Al^{3+} is the dominant aluminium species to compete with Ca^{2+} for cell wall binding sites. However, with decreasing pH, protons will also become competitors with Ca^{2+} for binding at carboxylic groups.

Although Al toxicity in time clearly causes a decrease in plant tissue Ca concentrations and an inhibition of root growth, in short term experiments correlation in time and location between Al accumulation, Ca uptake and root growth inhibition is generally poor. Aluminium can affect root elongation at very low Al concentrations without affecting Ca uptake (Ryan *et al.*, 1994). Even when a correlation is found at plant organ level it may be disposed of when the plant tissues are studied in more detail (Schofield *et al.*, 1998).

The lack of correlation between Ca uptake and Al accumulation in the root has led to a re-evaluation of the Ca-displacement theory as an explanation for growth inhibition. In cell wall extension, additional factors appear to be more important than pectin cross-linking, like the role of extensin with its high Al^{3+}-binding capacity (Kenjebaeva *et al.*, 2001), the functioning of expansins in acid growth (Cosgrove, 2001; McQueen-Mason, 1995), and the role of Ca^{2+} in synthesis and maintenance of the cell wall (Konno *et al.*, 1999; Rengel, 1992). Al accumulating in the apoplast may also form a toxic environment by increasing the availability of Al for binding to the plasma membrane, for interfering with membrane protein functioning and for entering the root symplast (Kinraide, 1998; Rengel, 1996).

So far, most experiments on Al-cell wall interactions have been performed on intact plants or whole root systems in which it is very difficult to distinguish between Al pools in different root compartments. One of the problems of studying Al accumulation in whole plants is, that the measurements include Al adsorption to cell wall polysaccharides, proteins and membrane phenolic groups, combined with cytoplasmic Al and possible $AlPO_4$ or Al-hydroxides precipitates in the apoplast, which may obscure a correlation (Tice *et al.*, 1992). Rengel and Reid (1997) found that in giant algae (*Chara corallina*), 99.99% of the Al accumulated in the cell wall but that desorption of intact organisms did not remove all cell wall-bound Al, leading to an overestimation of symplastic Al. A second problem in Al phytotoxicity studies in whole plants is that the pH in the root apoplast cannot easily be measured accurately and data are often based on pH measurements of the bulk solution. If the apoplast pH deviates from this bulk pH, the Al speciation will also differ from that in the nutrient or soil solution.

Studying Al^{3+} adsorption specifically to the cell wall compartment, in relation to ambient pH and Ca^{2+} concentrations, was the main objective of this study. To investigate the interaction between aluminium and the cell wall more closely under controlled and defined conditions, *in vitro* adsorption experiments were performed on isolated cell wall material from tomato (*Lycopersicon esculentum* L.) root. Aluminium and proton concentrations were varied to characterise the competition between aluminium, calcium and protons for cell wall binding sites.

Citrate and malate were used as examples of organic anions, which can form strong complexes with aluminium, a possible mechanism of Al tolerance in plants. A Gaines-Thomas exchange model was used to describe the competition for cell wall binding sites between Al, Ca and H.

Material and Methods

Plant material

Seeds of tomato (*Lycopersicon esculentum* L. cv. Moneymaker) were germinated in moist quartz sand at approximately 25°C under greenhouse conditions. After 13 days the seedlings were transferred to 150 L containers with nutrient solution consisting of (in mM): Ca (2.5), K (7.5), Mg (1.0), N (7.5: 2.0 as NH_4^+ and 5.5 as NO_3^-), P (0.6) and S (1.0), and trace elements (in μM): Fe (100), B (46), Zn (0.8), Mn (9), Cu (0.3) and Mo (0.1). Plants were grown for an additional 42 days in the greenhouse at 20°C.

At day 55 after sowing the roots were harvested, rinsed three times with demineralised water, blotted dry and, with the exception of the basal 5 cm of the roots, cut into 2-cm pieces. The total root mass was mixed and divided into portions of 10 g (fresh weight) which were stored at –18°C.

Isolation of root cell wall material

An adapted form of the procedure described by Sentenac and Grignon (1981) was used to isolate root cell wall. Samples of 10 g frozen root material were defrosted and rinsed for 90 min in 250 mL 0.4 M mannitol and 25 mM Tris-HCl solution (pH 7.4) under constant stirring. The root material was transferred to 150 mL solution containing 0.4 M sucrose, 25 mM Tris-HCl and 10 mM 2-mercapto-ethanol. Root fragments with a diameter larger than 2 mm were removed from the samples. The remaining material was laminated for 7 min by compression. The solution was replaced by 100 mL of a homogenisation solution consisting of 100 mM KCl, 5 mM Tris-HCl and 10 mM 2-mercapto-ethanol. The root material was homogenised for 20 sec using a blender and subsequently washed twice with fresh homogenisation solution. In between isolation steps the root material was wrung in cheesecloth to remove surplus solution. Isolation and subsequent adsorption experiments were done at room temperature.

Cell wall saturation with Ca^{2+}

After the last isolation step the cell wall material was rinsed three times with 10 mM HCl to remove adsorbed cations from negatively charged binding sites. To replace the protons at these binding sites with Ca^{2+}, the cell wall material was incubated 10 times for 5 min in 200 mL 10 mM $CaCl_2$ (pH 5.4 ± 0.1). Starting with the last acid rinse, the pH of the rinsing solutions was measured to monitor proton-calcium exchange. Ca^{2+} adsorbing on the cell wall material causes proton release and a subsequent decrease in pH of the incubation solution. As soon as Ca saturation of the binding sites is reached, at a given pH and calcium concentration, the pH of the solution will remain unchanged.

Al adsorption experiments

Adsorption experiments were carried out with Ca-saturated cell wall material, prepared as described above. Three sets of experiments were performed with emphasis on different aspects of Al adsorption and Al-Ca competition: (1) dependency on the Al^{3+} activity of the adsorption solution ($\{Al^{3+}\}_{sol}$); (2) pH dependency and (3) the influence of citric or malic acid on Al adsorption. Ca adsorption at 0 μM Al concentration was used as a control for Ca adsorption capacity of the cell wall at a given pH. Total Ca concentration in the solution ($[Ca]_{sol}$) was kept constant at 1 mM.

In experiment 1 adsorption solutions contained an aluminium concentration of 0, 25, 50, 75, 100 or 200 μM. Experiments were performed at pH 4 and 3.5 for comparison of pH effects at different Al levels.
For experiment 2 the solution concentration was kept constant at 100 μM Al and 1 mM $CaCl_2$. Here the pH was varied: pH 3, 3.5, 3.75, 4 and 4.25, respectively.

The third and final set of experiments was similar to exp. 2, with $[Al]_{sol}$ = 100 μM, but with the addition of 100 μM citric acid as an Al-complexing agent. The pH range was set at 3.5 to 4.25. For comparison with a weaker chelator, the effect of adding 100 μM malic acid at pH 3.5 and pH 4 was studied. Experiments were performed in duplicate. As treatments with 0 and 100 μM Al at pH 4 and 3.5 were used as reference, more data were collected for these treatments.

In all experiments, incubation solutions were refreshed eight times during a total adsorption time of 135 min: one 30-min incubation in 500 mL adsorption solution, followed by seven 15-min washes in 200 mL of the same adsorption solution. Replacement of the adsorption solutions ensured a constant ionic composition and pH during adsorption. The pH of the solutions was monitored during the incubations and the first and last adsorption solutions were sampled. After the last adsorption rinse the cell wall material was dried overnight at 70°C. Each sample of the dry cell wall material was weighed and desorbed for 90 min in 100 mL 0.1 M HCl under constant shaking. Concentrations of Al and Ca in the adsorption and desorption solutions were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES). *In vitro* Al and Ca adsorption to cell wall material ($[Al]_{cw}$ and $[Ca]_{cw}$, respectively) were expressed in μmol cation per g DCW (dry cell wall) or in milli-equivalent (meq) g DCW^{-1}. A correction for the contamination of the samples with incubation solution was calculated following the procedure by Sentenac and Grignon (1981). Accumulation of Al on the cell wall surface as a result of precipitation of Al-hydroxides is not expected at this low $[Al]_{sol}$ and pH range.

Cation exchange model

Cation sorption of H^+, Ca^{2+} and Al^{3+} on the isolated cell wall is described as a multi-component exchange process, with the cations competing for the same binding sites. Initial exchange coefficients K^{GT} are derived from the measured data, conform the Gaines-Thomas convention, using H^+ as reference ion. According to this convention, the exchange of bound H for Ca can be described as:

$$1/2\ Ca^{2+} + SH \leftrightarrow SCa_{1/2} + H^+,$$

with exchange coefficient:

$$K^{GT}_{Ca\backslash H} = [(E_{Ca})^{1/2} * \{H^+\}]\ [E_H * \{Ca^{2+}\}^{1/2}]^{-1}, \quad (1)$$

and exchange of H for Al:

$$1/3\ Al^{3+} + SH \leftrightarrow SAl_{1/3} + H^+$$

$$K^{GT}_{Al\backslash H} = [(E_{Al})^{1/3} * \{H^+\}]\ [E_H * \{Al^{3+}\}^{1/3}]^{-1}; \quad (2)$$

{cation^{a+}} denotes activity in the solution of a cation with valency a. E_{Al}, E_{Ca} and E_H are equivalent fractions of bound cations, relative to the total number of adsorption sites, Q_{max}, in meq (g DCW)$^{-1}$, with

$$E_{cat} = a*[cation]_{cw} (Q_{max})^{-1} \quad (3)$$

and

$$E_{Al} + E_{Ca} + E_H = 1. \quad (4)$$

Eq 4 indicates that the model assumes that all cell wall adsorption sites are occupied by cations and that the charge of the binding sites is fully neutralised by the adsorbed cations. The Gaines-Thomas model also assumes that all binding sites have the same chemical behaviour, i.e. it is a homogeneous exchange model. The value for the total number of cell wall adsorption sites, Q_{max}, was estimated at 0.66 meq (g DCW)$^{-1}$ by potentiometric acid-base titration in a pH range of 3 to 10.5 (Nell Romanova, WUR, unpublished data).

From a combination of equation 1 and 2, the exchange coefficient for Ca\Al can be derived:

$$1/3\ Al^{3+} + SCa_{1/2} \leftrightarrow SAl_{1/3} + 1/2\ Ca^{2+}$$

$$K^{GT}_{Al\backslash Ca} = (K^{GT}_{Ca\backslash H})^{-1} * K^{GT}_{Al\backslash H} \quad (5)$$

Al^{3+} activity in the adsorption solutions ($\{Al^{3+}\}_{sol}$), depending on $[Al]_{sol}$, solution pH and the presence of citric or malic acid anions, was calculated using ECOSAT (Equilibrium Calculation Of Speciation And Transport; Keizer *et al.*, 1992). ECOSAT was also used to optimise the Gaines-Thomas exchange coefficients for cell wall adsorption, $K^{GT}_{Al\backslash Ca}$ and $K^{GT}_{Ca\backslash H}$. Preliminary coefficient values, calculated using the above mentioned equations were used as input parameters, together with calculated solution activities of Al^{3+} and Ca^{2+}, pH and adsorption data.

Results

Ca-Al-H exchange: experimental results

Al and Ca adsorption at different external Al concentrations (exp. 1)

Aluminium adsorption to isolated tomato root cell wall ($[Al]_{cw}$) increased with increasing Al^{3+} activity in the solution (fig. 1, table 1). With this rise in Al adsorption, Ca adsorption from the solution onto the cell wall material ($[Ca]_{cw}$) decreased gradually to approximately 20% of control levels (–Al), at external Al concentrations > 100 μM. At 200 μM Al, $[Al]_{cw}$ appeared not to have reached its maximum value yet.

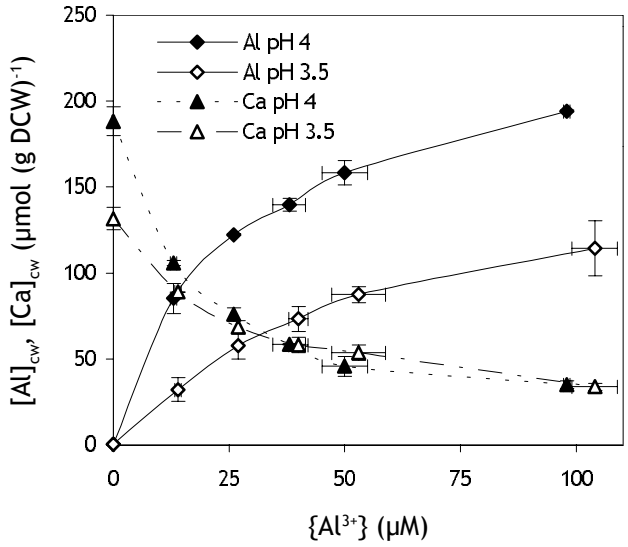

Figure 1: *Al and Ca adsorption to isolated root cell wall in relation to Al^{3+} activity ($\{Al^{3+}\}_{sol}$) and pH in the solution. Open and closed symbols represent pH 3.5 and 4.0, respectively (exp. 1).*

Performing the experiments at pH 3.5 lowered the adsorption levels of Al over the whole pH range. This pH effect was relatively strongest at a low Al solution concentration, when the Al^{3+} $(H^+)^{-1}$ ratio is lowest: lowering the solution pH by half a unit decreased $[Al]_{cw}$ by >60% at $[Al]_{sol}$ = 25 μM, compared to a decrease of 40% at $[Al]_{sol}$ = 200 μM. The pH change had a smaller effect on $[Ca]_{cw}$ (fig. 1). Although Ca adsorption was lowered by one-third when the pH was brought down from 4 to 3.5 in the absence of Al, it was less affected by the pH with increasing $[Al]_{sol}$.

	$\{Al^{3+}\}_{sol}$ (μM)	
$[Al]_{sol}$ (μM)	pH 3.5	pH 4
0	0	0
25	14	13
50	27	26
75	40	38
100	53	50
200	104	98

Table 1: *Al^{3+} activity ($\{Al^{3+}\}_{sol}$) in an aqueous solution of 1 mM $CaCl_2$, at pH 3.5 and 4, respectively. The difference between the total Al concentration and the activity of dissociated Al^{3+} in the solution is mainly caused by the activity coefficient of 0.52 for Al^{3+}. The presence of Al-hydroxides is a minor factor at this low pH.*

pH dependency of Al and Ca adsorption (exp. 2)

When the $[Al]_{sol}$ was kept at 100 μM and the pH range was extended to pH 3 – 4.25 in the second set of experiments, Al adsorption increased linearly with increasing pH over the whole range (fig. 2A, closed diamonds). Ca adsorption of the control treatments (without Al) and pH also showed a strong positive linear relationship (fig. 2B, open triangles). The Ca adsorption increase per pH unit was smaller than for Al adsorption.

When $[Al]_{cw}$ was plotted against $[Ca]_{cw}$, values for pH 3.5 and 4 were linearly correlated, with a correlation coefficient of –0.98 and –0.99, respectively (fig. 3); the molar Al\Ca exchange ratio was 1 : 0.9.

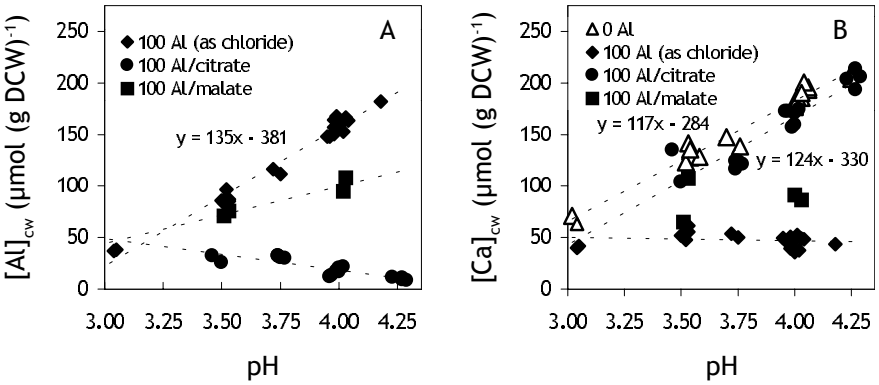

Figure 2: *pH dependency of (A) Al and (B) Ca adsorption to isolated root cell wall. Different symbols represent the different treatments: control (0 Al, open triangles) and 100 µM $AlCl_3$, with no added organic anion (closed diamonds), 100 µM citrate (closed circles) and 100 µM malate (closed squares), respectively (exp. 2).*

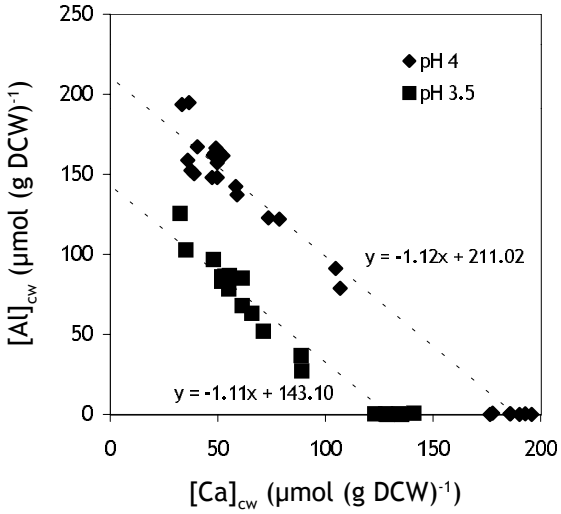

Figure 3: *Al adsorption to isolated root cell wall material in relation to adsorbed Ca at pH 3.5 (squares) and pH 4.0 (diamonds).*

Adsorption of Al and Ca: effect of organic anions citrate and malate (exp. 3)

In this experiment aluminium and citric or malic acid were added in equimolar concentrations to the solutions to form an Al-organic acid complex. Complexation by an organic anion decreases Al activity in the solution, with the largest effect at higher pH (table 2).

pH	$\{Al^{3+}\}_{sol}$ (µM)		
	$AlCl_3$	Al + citrate*	Al + malate**
3	53	36	32
3.25	54	24	24
3.5	53	14	17
3.75	53	8	12
4	50	5	9
4.25	46	3	6

Table 2: *pH dependency of Al^{3+} activity ($\{Al^{3+}\}_{sol}$) in an aqueous solution of 100 µM $AlCl_3$ and 1 mM $CaCl_2$, and with addition of 100 µM citric or malic acid, respectively. The difference between the total Al concentration and the activity of dissociated Al^{3+} in the solution is at low pH mainly caused by the activity coefficient of 0.52 for Al^{3+}. The concentration of Al-hydroxides in the solution increases from < 1% at pH 3 to 18% of total Al concentration at pH 4.25.*

* : *formation constants for Al reactions with citrate from Blamey et al. (1997), and Lindsay (1979).*

** : *close approximation of $\{Al^{3+}\}$, since the formation constant for Al-malate, taken from Nordstrom and May (1989), was determined at 20°C, instead of the standard 25°C. Formation constants for mal from Martell and Smith (1979).*

Using part of the same pH range as in exp. 2 and comparing adsorption of Al influenced by citrate-complexation with that of $AlCl_3$, citrate addition decreased $[Al]_{cw}$ by 63% at pH 3.5 to 94% at pH 4.25 (fig. 2A, closed circles). In the presence of Al-citrate Ca adsorption in this experiment reached values of 85% of control $[Ca]_{cw}$ levels at pH 3.5 to 94% at pH 4.25 (fig. 2B, closed circles).

Performing the experiment at pH 3.5 and 4 with 100 μM Al-malate instead of Al-citrate resulted in a limited prevention of Al adsorption. Relative to the control (+Al), $[Al]_{cw}$ was decreased by 16% (pH 3.5) and 37% (pH 4), respectively. $[Ca]_{cw}$ was reduced to 65% and 49% of its control level at pH 3.5 and pH 4, respectively (fig. 2A and 2B, closed squares).

Adsorption solution pH

It was important to limit the pH change of the adsorption solutions in order not to influence Al-speciation and cation competition. In the adsorption experiments, the pH of $AlCl_3$-containing solutions decreased at each rinse, with a maximum deviation of –0.12 pH unit at the first rinse, indicating proton release from the cell wall. In –Al and in Al-organic anion treatments the pH increased at the first rinse with 0.3 and 0.2 units, respectively. Deviations from the pH of the original adsorption solutions at the final incubations were on average ± 0.03 for Al-free solutions and ± 0.02 for Al-containing solutions but never larger than 0.07 pH unit.

Ca-Al-H exchange: Gaines-Thomas exchange model

For a first estimation of exchange coefficients for Ca\H and Al\H, the intersects of the trendline for pH 3.5 with the x- and y-axes in fig. 3 were taken as the adsorption of Ca at $[Al]_{sol} = 0$ and of Al at $[Ca]_{sol} = 0$, respectively. Multiplied by their valency gives the adsorption in meq $(g\ DCW)^{-1}$. For an approximation of the solution activity, an average is taken of the calculated activity values for all samples at pH 3.5; activity is given in mol L^{-1}. Input parameters and K_{GT} values, calculated according to eq 1, 2 and 5, are collected in table 3.

Q_{max}	$\{H^+\}$	$\{Ca^{2+}\}$	$\{Al^{3+}\}$	$[Ca]_{cw}$	$[Al]_{cw}$	$[H]_{cw}$	K^{GT}_{CaH}	K^{GT}_{AlH}	K^{GT}_{AlCa}
0.66	$10^{-3.53}$	$10^{-3.08}$	-	0.258	-	0.402	$10^{-1.98}$	$10^{-1.70}$	1.91
	$10^{-3.52}$	-	$10^{-4.28}$	-	0.429	0.231			

Table 3: *Input parameters for and a preliminary value of the Gaines-Thomas coefficients for exchange of Al, Ca and H on isolated root cell walls. Solution activities are given in mol L^{-1}, Q_{max} and cell wall adsorption are in meq $(g\ DCW)^{-1}$.*

Fitting the data with the acquired values for $K^{GT}_{Al\backslash Ca}$ and $K^{GT}_{H\backslash Ca}$ ($= (K^{GT}_{Ca\backslash H})^{-1}$), using Ca^{2+} as the reference ion, rendered optimised values for the exchange coefficients of $K^{GT}_{Al\backslash Ca} = 1.6$ and $K^{GT}_{H\backslash Ca} = 140$ and a Q_{max} of 0.74 meq (g DCW)$^{-1}$. Measured data plotted against calculated values showed that the model described the Al adsorption very accurately and the Ca adsorption reasonably well (fig. 4A and B).

When all data points for Al adsorption at the different pHs are plotted against the ratio of solution activities $\{Al^{3+}\}\{H^+\}^{-1}$, the curves from fig. 1 fuse into one curve (fig. 5A). Calculated Ca^{2+} activities for these points were reasonably constant at 0.83 ± 0.05 mM, over the whole pH range. The exchange model, with the use of the above-mentioned parameters, could again describe the data, though the model somewhat underestimated the Al adsorption at higher $\{Al^{3+}\}\{H^+\}^{-1}$ ratio. The fit was better when a small adjustment was made, by raising the Q_{max} to 0.80 while keeping the same K^{GT} values (fig. 5B).

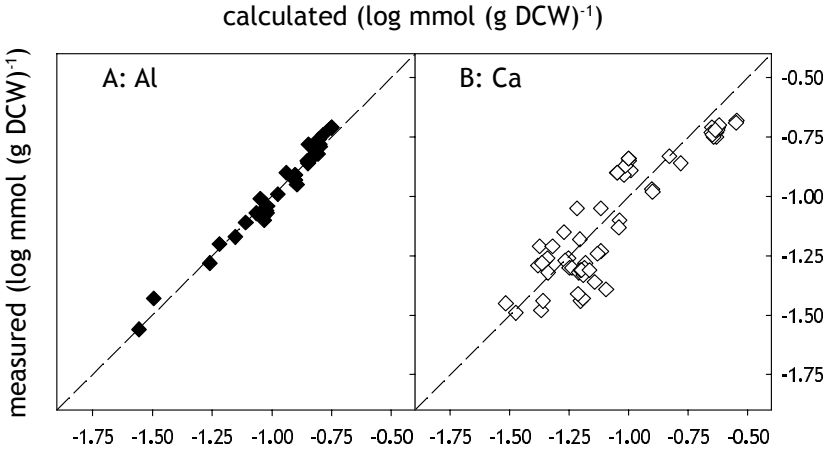

Figure 4: *Plot of measured values against calculated values for (A) Al and (B) Ca adsorption to isolated root cell wall. Dashed line is a reference line of every calculated values against itself.*

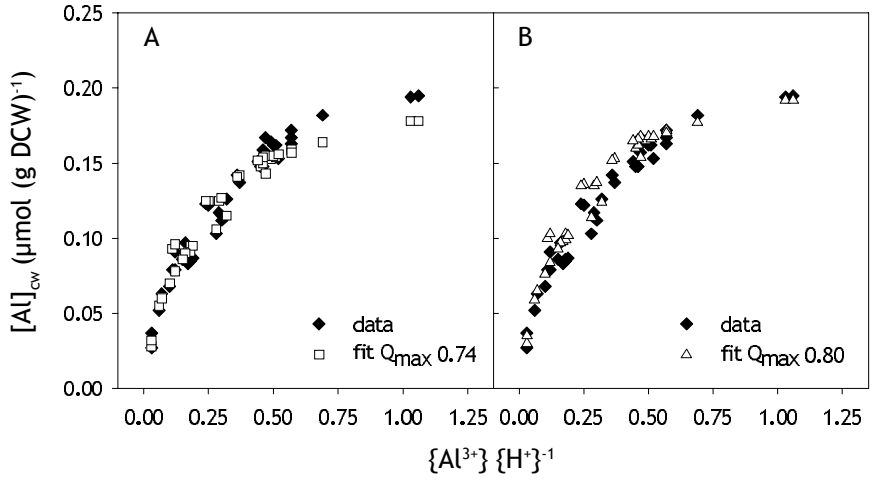

Figure 5: *Plot of measured and calculated values for Al adsorption against the ratio of Al^{3+} activity H^+ activity^{-1}. Maximum number of adsorption sites, Q_{max}, is set at (A) 0.74 or (B) 0.80 meq (g DCW^{-1} (dry cell wall).*

With these K^{GT} values and the three different values for Q_{max} (0.66, 0.74 and 0.80 meq (g DCW)$^{-1}$), a plot of calculated Al adsorption against pH, over the measured data for Al adsorption from fig. 2, shows a slightly better fit with Q_{max} = 0.66 meq (g DCW)$^{-1}$ at a pH < 3.75 and the best fit with the larger Q_{max} values at higher pH (fig. 6). A Q_{max} of 0.74 meq (g DCW)$^{-1}$ is accepted as the best overall fit for Al adsorption. Calculated Ca adsorption in the presence of Al at Q_{max} = 0.74, was slightly too low at low pH and increased too steeply with increase in pH (fig. 7, +Al Q_{max} 0.74).

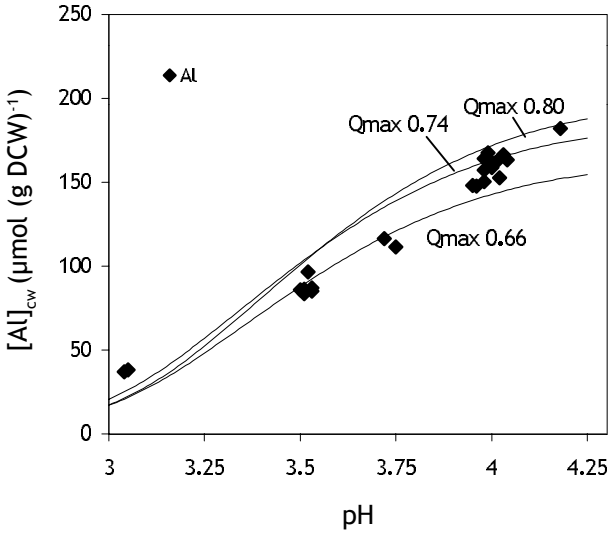

Figure 6: *Simulation of Al adsorption to isolated cell wall, from a solution containing 100 µM $AlCl_3$ and 1mM $CaCl_2$, at different pH levels. Calculated values and measured data are set against the pH. Maximum number of adsorption sites, Q_{max}, is set at 0.66, 0.74 or 0.80 meq (g DCW)$^{-1}$; $K^{GT}_{H\backslash Ca}$ = 140, $K^{GT}_{Al\backslash Ca}$ = 1.6. Data points are taken from fig. 2A.*

Ca adsorption in the absence of Al, as described by the top line in fig. 2B is, however, not so well described when using Q_{max} = 0.66 meq (g DCW)$^{-1}$ and $K^{GT}_{H\backslash Ca}$ = 140 (fig. 7, –Al Q_{max} 0.74). Ca adsorption is severely underestimated at low pH and overestimated at pH > 3.75. Decreasing the total Ca binding capacity to 0.45 meq (g DCW)$^{-1}$, which reduced the exchange coefficient from 140 to approximately 50, gave a much better estimation of the Ca adsorption in relation to pH (fig. 7, Q_{max} 0.45).

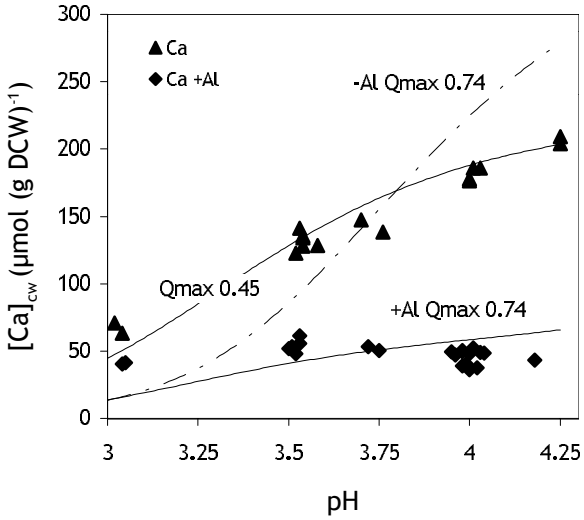

Figure 7: *Simulations of Ca adsorption to isolated cell wall, from a solution containing 0 or 100 µM $AlCl_3$ and 1mM $CaCl_2$, at different pH levels. Calculated values and measured data are set against the pH. Model parameters (Q_{max}, $K^{GT}_{H\backslash Ca}$, $K^{GT}_{Al\backslash Ca}$) for the different simulation are set at: (0.74, 140, -) and (0.45, 50, -) for Ca (triangles) and at: (0.74, 140, 1.6) for Ca + Al (diamonds). Data points are taken from fig. 2B.*

When including formation constants for Al-citrate in the calculation and using the same parameters (K^{GT} and Q_{max}) as for the calculation of Al adsorption without organic anion, the model described values, which deviated from the measured data. Al adsorption to the cell wall from a solution containing Al and citric acid was severely overestimated for most data points and subsequently Ca adsorption was underestimated in most samples (data not shown). However, when the same $K^{GT}_{H\backslash Ca}$ and Q_{max} values were used as for Ca adsorption (50 and 450, respectively), resulting in an estimated value for $K^{GT}_{Al\backslash Ca}$ of 1, Al and Ca adsorption could be very well described by the Gaines-Thomas exchange model (fig. 8, Al/cit and fig. 9, Ca/cit, respectively). Though described Al adsorption in the presence of malate approached the measured data, the Ca adsorption was overestimated at higher pH (fig. 8 and 9, Al/mal and Ca/mal, respectively). Insufficient data points were available for a reasonable verification of the model.

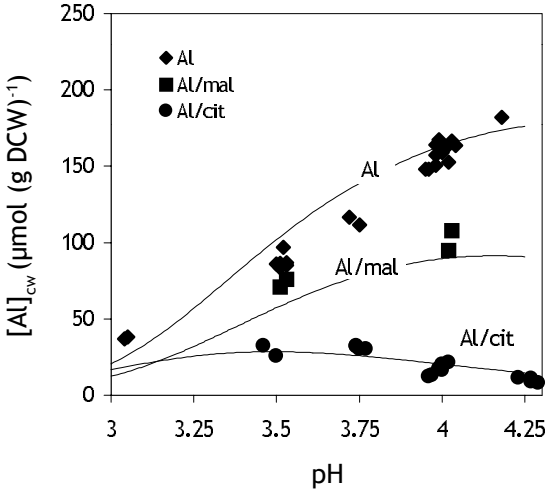

Figure 8: *Simulations of Al adsorption to isolated cell wall, from a solution containing 100 µM $AlCl_3$, 1mM $CaCl_2$ and with addition of 0 organic anion (Al), 100 µM citrate (Al/cit) or 100 µM malate (Al/mal), at different pH levels. Calculated values and measured data are set against the pH. Model parameters (Q_{max}, $K^{GT}_{H\backslash Ca}$, $K^{GT}_{Al\backslash Ca}$) for the different simulation are set at: (0.74, 140, 1.6) (Al), (0.45, 50, 1) (Al/cit) and (0.74, 140, 1.6) (Al/mal), respectively. Data points are taken from fig. 2A.*

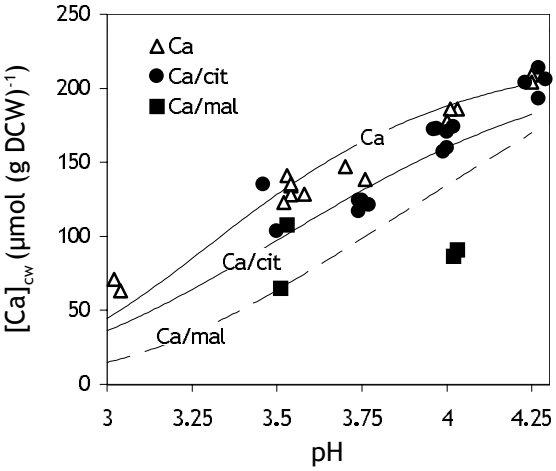

Figure 9: *Simulations of Ca adsorption to isolated cell wall, from a solution containing 1 mM $CaCl_2$ (Ca) or 100 µM $AlCl_3$ and 100 µM citrate (Al/cit) or 100 µM malate (Al/mal), at different pH levels. Calculated values and measured data are set against the pH. Model parameters (Q_{max}, $K^{GT}_{H\backslash Ca}$, $K^{GT}_{Al\backslash Ca}$) for the different simulation are set at: (0.45, 50, -) (Ca), (0.45, 50, 1) (Al/cit) and (0.74, 140, 1.6) (Al/mal), respectively. Data points are taken from fig. 2B.*

Discussion

With increasing Al^{3+} activity in the solution, $[Al]_{cw}$ increases and $[Ca]_{cw}$ decreases in a pH dependent manner. The slight decrease in pH, observed in the adsorption solutions at every rinse, indicates that protons are released, together with Ca^{2+} ions. There are two options for Ca-Al exchange on the cell wall to satisfy demands of charge-neutrality. For the first option, Al^{3+} adsorption has to be accompanied by desorption from the cell wall of additional cations, (i.e. protons) besides Ca^{2+}, in order to create sufficient negative binding sites for the aluminium and keep total charge unaltered. The second option would be binding of Al in the form of $AlOH^{2+}$, causing subsequent proton release by hydrolysis to form new $AlOH^{2+}$ and restore equilibrium in the solution. Which of the two options applies here could not immediately be concluded from the data, since both include an Al\Ca exchange and proton release. Both options have been explored in the Gaines-Thomas exchange model used here and adsorption calculations for both Al^{3+} and $AlOH^{2+}$ have been made. Using $AlOH^{2+}$ activity in the exchange model proved to be insufficiently sensitive to the pH changes to describe the Al adsorption in any way and was no longer taken into consideration.

With Al^{3+} as the adsorbing Al species, the model described the adsorption of Al very well. Ca adsorption could also be quite well described, after adjustment of the parameters for the total number of adsorption sites and the Gaines-Thomas exchange coefficients. The initial value for Q_{max} of 0.66 meq $(g\ DCW)^{-1}$ was based on titration over a wide pH range. This means that not only the carboxylic groups of the cell wall pectin were taken into account but also dissociating groups at higher pH. The Q_{max}, resulting from the data on Ca adsorption in the absence of Al, is more likely to represent the cation exchange capacity (CEC) of the pectic groups in the cell wall. The Q_{max} for Al adsorption was substantially larger than the value for pectic CEC would allow and even slightly larger than the value found by titration. Non-carboxylic binding sites could be $-NH_2$ or -SH groups of cell wall proteins or phenolic -OH groups, with a $pK > 7$ (Allan and Jarrell, 1989; Meychik and Yermakov, 2001). One likely candidate for non-carboxylic Al binding is extensin, a cell wall protein which has a high binding capacity for Al (Horst, 1995; Kenjebaeva *et al.*, 2001).

Additionally, Al may cause conformational changes in (cross-links between) cell wall components or in adhering proteins, creating new binding sites (Caldwell, 1989; Carpita and Gibeaut, 1993). The non-carboxylic binding sites may play a role in Al binding to the cell wall but are not expected to play in role in Ca binding within the pH range of the experiments. This mechanism may explain the apparent difference in maximum site densities needed to describe the data optimally.

If additional Al adsorption sites are very different from the pectic carboxylic groups, the K^{GT} values derived here for Al exchange must be regarded as a combined exchange coefficient for all groups. Further analysis of Al adsorption to plant cell walls may lead to new exchange coefficients, one for each separate binding site, which could be included in a more heterogeneous adsorption model, like the NICA (non ideal competitive adsorption) model. This model has been used by Plette *et al.* (1996) to describe competitive binding of Cd, Zn, Ca and protons to cell walls of a gram-positive bacterium. The bacterial walls contained both carboxylic and amino-type binding sites. Changing the total number of binding sites also increased the $K^{GT}_{H\backslash Ca}$ and thus competition between Ca and H for the carboxylic groups, in favour of H. When Al is bound to the cell wall, the monovalent protons are apparently less hindered in binding to the surface than the divalent Ca ions are. Rengel and Robinson (1989a), who showed a relative increase in K^+ and Na^+ adsorption in *Lolium multiflorum* L. as a result of Al exposure, have reported a similar phenomenon.

The data presented here show that, *in vitro*, Al replaces Ca at the cell wall binding sites to a large extent. The Al\Ca molar exchange ratio of 1:0.9 is markedly higher than the value of Al:Ca = 1:1.65 found for Al:Ca exchange on Ca-pectate, one of the major Ca^{2+}-binding pectic components of the root cell wall (Blamey and Dowling, 1995; Ostatek-Boczynski *et al.*, 1995). Pectin in solution behaves differently from cell wall pectin, which could cause a discrepancy between results for adsorption to cell walls and pectate (Carpita and Gibeaut, 1993). The Al:Ca ratio is also higher than would be expected on the basis of a charge-neutral exchange with a ratio of 1:1.5, when only Al and Ca exchange are taken into account. The curves for Al and Ca (–Al) adsorption in figures 8 and 9 appear to be identical, which would mean an

identical pH dependency of adsorption of Al and Ca. This is in sharp contrast with a higher affinity of cell walls for trivalent cations and also with the relative pH independence of Al accumulation found for *Chara corallina* cell walls (Taylor *et al.*, 2000). Al adsorption to tomato cell wall material proved to be even slightly more sensitive to pH changes in the range of pH 3 to 4.25 than Ca adsorption. This would mean that, under these circumstances, adsorbed Al would be at least as easily removed by apoplast acidification as adsorbed Ca is. Less easily reversed cross-linking of the pectic matrix by Al, leading to increased rigidity of the cell wall, would under these circumstances not be the restricting factor for cell elongation in tomato roots. This is in concordance with current views that inhibition of root elongation by aluminium involves more than replacing calcium at pectic cross-links.

However, the adsorption curves and the exchange ratios are based on the total Al adsorption to the isolated cell wall, including both carboxylic and other binding sites. Assuming that Al displaced protons and not Ca from the non-carboxylic sites leaves less Al to replace the measured amount of Ca, decreasing the Al:Ca exchange ratio. This also decreases the pH dependency of the Al adsorption, with a decrease in the slope of the Al^{3+} adsorption in figure 2A. Additionally, an equal or higher pH dependency for Al^{3+} adsorption than for Ca^{2+} would not be conform the exchange model proposed here: Al displaces one Ca ion and one proton on the cell wall, whereas Ca exchanges against two protons.

The pH dependency of Al adsorption to the cell wall material implies that a high [Al^{3+}], as found in increasingly acid mineral soils, does not immediately imply high Al accumulation in plant root cell walls. Whether the competition mechanism between Al^{3+} and H^{+}, resulting in high cell wall proton adsorption, will reduce the negative effects of extreme soil acidity is improbable. Under such acid conditions H^{+} replaces Al^{3+} as major cause of cell wall cation-displacement and growth inhibition (Kidd and Proctor, 2001; Koyama *et al.*, 2001).

At the low Al activity in the presence of citric acid, the Q_{max} of the cell wall is not increased, indicating that under these circumstances only the pectic binding sites are occupied by Al and Ca. Low levels of Al adsorption from the Al-citrate solution can be explained by free Al^{3+}, which is present even

when Al and citrate are present in a 1:1 molar ratio. The Ca adsorption can be described with the exchange parameters for Ca adsorption without Al and the difference between the Ca adsorption –Al and that of + Al/citrate can be fully covered by the low-level adsorption of Al on the cell wall.

The effect of malate is less well defined with the model, although Al^{3+} activity according to speciation calculation should be almost as much reduced as with citrate. The discrepancy between Al^{3+} activity and Al adsorption may be attributed to an inadequate formation constant for Al-malate. The only available constant was not determined at the standard 25°C and may cause a deviation in the results. Other Ca- and Al-malate species have been mentioned in literature but adequate constants were not always available. Using a rough estimation of these constants did not give results that differed substantially from the ones mentioned here and the additional species were left out of the calculations.

High proton concentration lead to a diminished formation of Al-citrate complexes in the results presented here, resulting in a slight elevation in Al adsorption at the lowest pH level. Superimposed on this higher availability of Al^{3+} for cell wall adsorption at extremely low pH is the increasing competition by protons for the binding sites. Exudation of organic anions, like citrate, malate and oxalate, is considered to be a plant tolerance mechanism for aluminium. Whole plant studies with maize, using different organic acids for aluminium complexation show a variable decrease in Al-toxicity (Keltjens, 1995). Blamey *et al.* (1997) strongly decreased Al^{3+} sorption on Ca-pectate in *in vitro* experiments by adding citrate; malate proved to be less effective. Prevention of aluminium interaction with root compartments like the cell wall, through complexation of the toxic metal, forms an obvious explanation for aluminium detoxification. However, a low pH decreases the formation of Al-citrate and Al-malate chelates. This means that exudation of organic anions like citrate can be effective in preventing Al cell wall adsorption at a pH around 4 but it will lose its significance with decreasing pH.

3

Effects of aluminium on nutrient cation adsorption to isolated root cell walls of tomato; the role of Al-chelators citrate and EDTA.

Abstract

The plant root apoplast has a high capacity for binding cations, both nutrients as well as phytotoxic ions, like aluminium. Competition for adsorption to cell wall binding sites may be part of the reason nutrient uptake is decreased in Al-affected plants. *In vitro* competition of Al^{3+} and nutrient cations for cell wall binding sites in the plant root was investigated in roots of tomato (*Lycopersicon esculentum* L.). Isolated cell wall material was exposed to a nutrient solution, containing 0 or 100 μM $AlCl_3$. The effect of Al complexation on its adsorption and competition behaviour at binding sites was studied by adding various concentrations of the Al-chelators citrate and ethylenediamine tetraacetic acid (EDTA). Al adsorbed strongly to the cell wall, while significantly decreasing the adsorption of Ca, Mg, Zn and Cu. The decrease in Ca, Mg and Mn adsorption was linearly related to the increasing Al adsorption; Zn showed a similar relationship in the absence of EDTA. Complexation of Al prevented it from competing with the nutrient cations for adsorption, though this effect was only complete when Al activity in the solution had been reduced by 90%.

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Introduction

The root apoplast is the first compartment a soil solution encounters upon entering a plant root. In the earlier days of plant biology this continuous space outside the cell membranes was considered to solely consist of an inert, rigid cell wall. At present, the apoplast is known to be a metabolically active complex of cell walls, proteins and the outer surface of the plasma membrane (Carpita and Gibeaut 1993; Sattelmacher, 2001).

Nutrients from the soil solution can adsorb to the surfaces in the apoplast and can from there be transported over the membrane, into the cells (Haynes, 1980; Sattelmacher, 2001). Negatively charged carboxylic groups of the cell wall pectin are the main contributors to cation adsorption, followed by acidic amino acids of proteins (Meychik and Yermakov, 2001; Richter and Dainty, 1989). The carboxylic binding sites show large differences in affinity for the different cations, based on valency and ionic radius. Trivalent cations, like the monomeric aluminium species Al^{3+}, will be more strongly attracted than divalent nutrient cations (Parker *et al.*, 1998; Richter and Dainty, 1989; Sentenac and Grignon, 1981). Although affinity for monovalent cations is usually low, proton affinity of the sites is high and the cell wall can to some extent function as a buffer for apoplast pH under mildly acid conditions (Mizuno and Katou, 1992; Parker *et al.*, 1998).

A soil solution from an acid mineral soil (pH < 4.5) may contain phytotoxic concentrations of monomeric aluminium species, while at extremely low pH and in organic soils, proton toxicity will become more important (Keltjens, 1997; Kidd and Proctor, 2001; Marschner, 1991). Al^{3+} can accumulate in cell walls of the plant root cortex and epidermis, especially in root tips of newly formed roots (Blamey, 2001; Ryan *et al.*, 1997). Phytotoxic Al concentrations may influence nutrient uptake directly by competing with nutrients for uptake by cation channels in the plasma membrane or by interfering with the channel functioning (Liu and Luan, 2001; Rengel *et al.*, 1995; Yamamoto *et al.*, 2001). High Al concentrations close to the plasmalemma are also expected to increase Al uptake, although it is still unclear if Al binding to the cell wall is a prerequisite for such uptake (Kochian, 1995; Zhang and Taylor, 1990).

The extent of Al toxic effects on nutrient uptake will depend on whether the plant species has a mechanism to resist or avoid toxic Al concentrations (Marschner, 1991; Keltjens, 1997). One resistance mechanism is the exudation of organic ligands, like low molecular weight organic acids or phenolics, which can bind metals and cause shifts in their free ionic concentrations. Citrate is one of the major Al-chelators in Al resistant plants growing on acid soils. Transport of the neutral Al-citrate complex over the cell membrane is limited, thus forming a mechanism for the plant to prevent high internal Al concentrations (Kidd *et al.*, 2001; Ma *et al.*, 2001; Ostatek-Boczynski *et al.*, 1995; Piñeros *et al.*, 2002; Silva *et al.*, 2001c).

The function of the cation cell wall adsorption in plant nutrition is still a matter of debate. Creating relatively high nutrient concentrations in the vicinity of the plasma membrane is considered to facilitate nutrient uptake, by forming an available metabolic pool for active transport over the plasmalemma (van Oene, 1998; Rengel, 1990; Sattelmacher, 2001; Thornton and Macklon, 1989). When adsorbed at a distance from the plasmalemma, direct interaction between the cell wall and the membrane ion channels may not be possible. Nutrient acquisition within the apoplast could in such circumstances be supported by a chelator like citrate, phytosiderophores and amines (Hart *et al.*, 1998; Krähmer and Sattelmacher, 1997; Zhang *et al.*, 1991), transporting cations between cell wall and plasma membrane. Citrate-chelation of Mn has been mentioned as a means of the plant to regulate its uptake (Varga *et al.*, 2000).

A linear relationship between apoplast adsorption and net nutrient uptake rates into the cell was established for Ca (Godbold and Jentschke, 1998; Rengel, 1989). Mg adsorption in the apoplast has shown a linear correlation with its uptake into the plant in ryegrass, yet in experiments with a combination of Al and Mg, the correlation varied from high to non-existent (Godbold and Jentschke, 1998; Keltjens, 1995; Rengel, 1990). Cu adsorbs strongly to organic ligands in the cell wall (Kochian, 1991; van Cutsem and Gillet, 1983) and adsorption in the apoplast contributed substantially to the apparent uptake of Cu by living roots of ryegrass (Iwasaki *et al.*, 1990; Thornton and Macklon, 1989). Little is known of the effect of Al on root adsorption and uptake of trace elements.

Since aluminium, nutrient cations and protons all compete for the same binding sites (Richter and Dainty, 1989), the actual effect of aluminium on nutrient cell wall adsorption will strongly depend on ionic concentrations, pH and the presence of metal chelators in the solution. Investigations into the distribution of aluminium and nutrient cations within the root system have mostly been done by desorption or chemical analysis of whole root systems or excised roots (Godbold and Jentschke, 1998; Keltjens, 1995; Thornton and Macklon, 1989; Zhang and Taylor, 1990). In other experiments, isolated cell wall material was used to study the behaviour of single cations in cell wall adsorption (Richter and Dainty, 1989; Ryan and Kochian, 1993; van Cutsem and Gillet, 1983).

The objective of this paper was to study the effect of Al adsorption to the root cell wall on nutrient cation binding to the same material. A second objective was to study the extent to which complexation of Al can prevent this effect. Isolated cell wall material of tomato (*Lycopersicon esculentum* L.) roots were exposed to nutrient solutions for *in vitro* adsorption studies of Al^{3+}, Al-complexes and nutrient cations. Nutrient solution conditions represented a complete but relatively poor, acid soil solution. The choice for tomato as an example for a dicotyledonous plant was based on its relative sensitivity to aluminium. Citrate and ethylenediamine tetraacetic acid (EDTA) were used to investigate the adsorption behaviour of Al and nutrient cations on the cell wall in the presence of an Al-chelator. Citrate is easily degraded in solution; therefore EDTA, as a strong and more stable artificial chelator, was used in a parallel set of experiments. The effect of the presence of aluminium and chelators on nutrient cell wall adsorption is discussed.

Material and Methods

Plant material

Seeds of tomato (*Lycopersicon esculentum* L. cv. Moneymaker) were germinated in moist quartz sand at approximately 25°C in a greenhouse. After 13 days, the seedlings were transferred to 150 L containers with nutrient solution consisting of (in mM): Ca (2.5), K (7.5), Mg (1.0), N (7.5: 2.0 as NH_4 and 5.5 as NO_3), P (0.6) and S (1.0), and trace elements (in μM): Fe (100), B (46), Zn (0.8), Mn (9), Cu (0.3) and Mo (0.1). Plants were grown for an additional 42 days in the greenhouse at 20°C. At day 55 after sowing the roots were harvested, rinsed three times with demineralised water, blotted dry and, with the exception of the thicker basal 5 cm of the roots, cut into 2 cm pieces. The total root mass was mixed and divided into portions of 10 g (fresh weight) which were stored at –18°C.

Isolation of root cell wall material

An adapted form of the procedure described by Sentenac and Grignon (1981) was used to isolate root cell wall. Samples of 10 g root material were defrosted and rinsed for 90 min in 250 mL 0.4 M mannitol and 25 mM Tris-HCl solution (pH 7.4) under constant stirring. The root material was transferred to 150 mL solution containing 0.4 M sucrose, 25 mM Tris-HCl and 10 mM 2-mercapto-ethanol. Root fragments with a diameter larger than 2 mm were removed from the samples. The remaining material was laminated for 7 min by compression. The solution was replaced by 100 mL of a homogenisation solution consisting of 100 mM KCl, 5 mM Tris-HCl and 10 mM 2-mercapto-ethanol. The root material was homogenised for 20 sec using a blender and subsequently washed twice with fresh homogenisation solution. In between isolation steps the root material was wrung in cheesecloth to remove surplus solution. Isolation and subsequent adsorption experiments were done at room temperature.

Cell wall saturation with Ca^{2+}

After the last isolation step the cell wall material was rinsed three times with 10 mM HCl to remove adsorbed cations from negatively charged binding sites.

To replace the protons at these binding sites with Ca^{2+}, the cell wall material was incubated 10 times 5 min in 200 mL 10 mM $CaCl_2$ (pH 5.4 ± 0.1). Starting with the last HCl rinse, the pH of the rinsing solutions was measured to monitor proton-calcium exchange. Ca^{2+} adsorbing on the cell wall material will cause proton release and a subsequent decrease in pH of the incubation solution. As soon as Ca saturation of the binding sites is reached, the pH of the solution will remain unchanged.

Adsorption experiments

Adsorption experiments comprised treatment of the Ca-saturated cell wall material with (1) a complete nutrient solution, (2) the same nutrient solution with 100 μM Al and (3) a solution equal to that in (2), plus increasing concentrations of aluminium-chelator citrate (3a) or EDTA (3b). For control of the effect of the chelators on other cations than Al, in each subgroup of group 3, an adsorption experiment was performed in nutrient solution with 100 μM chelator, without aluminium (treatments 3a1 and 3b1, respectively). The total resulted in 12 different treatments (table 1).

Treatment	Al (μM)	citric acid (μM)	EDTA (μM)	*n*
1	0	0	0	6
2	100	0	0	17
3 a1	0	100	0	2
a2	100	25	0	4
a3	100	50	0	3
a4	100	75	0	4
a5	100	100	0	6
3 b1	0	0	100	2
b2	100	0	25	2
b3	100	0	50	2
b4	100	0	75	2
b5	100	0	100	3

Table 1: *Treatment groups for adsorption of aluminium and nutrient cations on cell wall material; pH = 4*

The composition of the nutrient solution was chosen to represent a relatively poor soil solution (Falkengren-Grerup *et al.*, 1995; Pintro *et al.*, 1996); [P] was kept low to prevent $AlPO_4$ precipitation and pH was set at 4.
The nutrient solution consisted of (in μM): 500 $Ca(NO_3)$, 150 K_2SO_4, 100 $MgSO_4$, 2 NaH_2PO_4, 46 H_3BO_3, 10 Fe-EDTA, 9 $MnSO_4$, 0.8 $ZnSO_4$, 0.3 $CuSO_4$ and 0.014 $(NH_4)_6Mo_7O_{24}$.

Adsorption solutions were refreshed eight times during a total adsorption time of 135 min: one 30-min incubation in 500 mL adsorption solution, followed by seven 15-min washes in 200 mL of the same adsorption solution. Replacing the adsorption solutions ensured a constant ionic composition and pH. For the Al-nutrient competition experiment, 100 μM $AlCl_3$ was added to the nutrient solution, before setting the pH. In the third group of experiments a range of citric acid or Na_2-EDTA concentrations (0-25-50-75-100 μM) was added to the Al-containing nutrient solutions.

The pH of the solutions was monitored during the incubations and the first and last adsorption solutions were sampled. After the last adsorption rinse the cell wall material was dried overnight at 70°C. Each sample of the dry cell wall material was weighed and desorbed for 90 min in 100 mL 0.1 M HCl under constant shaking. This acid concentration had previously been tested for effectiveness in desorbing Al from cell wall material and had removed up to 99% of adsorbed Al.

Concentrations of Al and the nutrients Ca, Mg, K, Zn, Cu, Mn, Fe and P in the adsorption and desorption solutions were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES) and -mass spectrometry (ICP-MS). *In vitro* Al and nutrient adsorption to cell wall material ($[Al]_{cw}$ and $[nutrient]_{cw}$, respectively) is expressed as μmol cation (g DCW (dry cell wall))$^{-1}$. The procedure by Sentenac and Grignon (1981) was used to correct for the contamination of the samples with incubation solution.

All treatments were performed in duplicate. As treatments with 0, 100 μM Al and 100 μM Al/100 μM citrate were used as reference between experiments, more data were collected for these treatments. Significant differences in adsorption of cations were analysed with ANOVA Dunnett's T3 multiple comparison test (SPSS 8.0).

Activity of Al^{3+} and nutrient cations in the adsorption solutions, depending on solution composition and pH, was calculated using ECOSAT (Equilibrium Calculation Of Speciation And Transport) (Keizer *et al.*, 1992).

Results

Adsorption of Al and nutrient cations to root cell wall material

Ca formed, with a concentration of 165 μmol Ca $(g\ DCW)^{-1}$, the largest adsorbed fraction of divalent cations on the cell wall material in control treatment 1 (table 2). Compared to Ca, adsorption of the other divalent macronutrient cation, Mg, was markedly lower. K adsorption was minimal at 0-1.8 μmol $(g\ DCW)^{-1}$, though K was present in a relatively high concentration in the nutrient solution. Because of the low contribution of K to the adsorbed fraction, data for K adsorption were not taken into further consideration. Of the trace element cations, especially Cu and Zn adsorbed in relatively large amounts to the cell wall surface, when compared to their respective concentrations in the nutrient solution.

Al accumulated on the cell wall in treatment 2 (nutrient solution + 100 μM $AlCl_3$). At the same time, the adsorption of all nutrient cations, except Mn, decreased significantly, compared to treatment 1 (table 2). Adsorption of Mg and Zn was decreased to approximately 10%, while Ca and Cu were decreased to 20 and 50% of control treatment 1, respectively. Mn adsorption was on average much lower in the Al treatment but a significant difference with the control treatment could not be established, due to large variance in data in the latter.

Aluminium adsorption controlled by chelator concentration: the effect on nutrient cation adsorption

In the presence of equimolar concentrations (100 μM) of Al and citrate or EDTA, Al adsorption was reduced by 90% and 98%, respectively, compared to the 100 μM $AlCl_3$ treatment. Table 2 shows average values for adsorption of Al and the nutrient cations, measured in *n* samples. The adsorption of all nutrient cations was not significantly different from the control level, with the addition of 100 μM citrate (3a5). Ca and Zn adsorption already reached this level in treatment 3a4 (75 μM citrate), whereas Mg

adsorption was still significantly affected at this chelator concentration. Cu adsorption, which was significantly reduced in the presence of 100 µM Al, was not statistically different from the control value in all citrate treatments. Citrate in the nutrient solution without Al (3a1) tended to lower adsorption of Zn and Cu but values were not significantly different from the control (table 2). Adsorption of Ca and Mg was significantly different from the control in all EDTA treatments, except at 100 µM (3b5). EDTA in the solution had a very strong effect on Zn adsorption, reducing it to 3% in treatment 3b5 and to zero in the absence of Al (3b1). EDTA completely blocked Cu adsorption in all treatments (table 2).

		Adsorbed (µmol (g DCW)$^{-1}$)						
				citrate (3a)				100 cit (-Al) (a1)
NS cation	$[cation]_{NS}$ (µM)	NS (1)	100 Al (2)	25 (a2)	50 (a3)	75 (a4)	100 (a5)	
Ca	500	165	36*	52*	77*	125	152	164
Mg	100	10.48	0.96*	1.91*	2.81*	5.26*	7.91	11.02
Zn	0.8	0.80	0.10*	0.18*	0.27*	0.63	0.75	0.56
Mn	9.0	1.28	0.18	0.38	0.68	1.19	1.30	0.41
Cu	0.3	0.97	0.43*	0.68	0.68	0.72	0.61	0.69
Al	0/100	0.26	171	150	108	56	18	0
				EDTA (3b)				100 EDTA (-Al) (b1)
		NS (1)	100 Al (2)	25 (b2)	50 (b3)	75 (b4)	100 (b5)	
Ca	500	165	36*	44*	56*	84*	166	162
Mg	100	10.48	0.96*	1.53*	2.64*	3.65*	10.21	10.30
Zn	0.8	0.80	0.10*	0.10*	0.14*	0.18*	0.02*	0*
Mn	9.0	1.28	0.18	0.21	0.36	0.67	1.67	0.03
Cu	0.3	0.97	0.43*	0*	0*	0*	0*	0*
Al	0/100	0.26	171	171	134	92	3.7	0

Table 2: *Cation adsorption to isolated cell wall material in the presence of a complete nutrient solution (NS, control treatment), NS + 100 µM $AlCl_3$ or NS + 0/100 µM $AlCl_3$ and 0 - 100 µM citrate or EDTA; pH = 4. Treatment numbers according to table 1. Results are average values of n samples.* indicates a significant difference in adsorption at the 0.05 level between Al (+/-chelate) and control treatment (1)*

The increasing chelator concentrations added to the solutions with 100 μM $AlCl_3$ in treatment group 3 created a range of Al adsorbed to the cell wall material ($[Al]_{cw}$; fig. 1, A and B). The range extends from close to zero at maximum chelator concentration to 198 μmol Al (g DCW)$^{-1}$ in the absence of chelator. When these $[Al]_{cw}$ values (fig. 1, y-axes) were set against the concentrations of adsorbed nutrient cations found in the same samples (x-axes), the values of $[Ca]_{cw}$, $[Mg]_{cw}$ and $[Mn]_{cw}$ showed a strong negative linear correlation with the concurrent $[Al]_{cw}$ (fig 1, A 1-3 and B1-3). In the case of Mn and Al/citrate, the two outliers, indicated by the circle and for which no obvious explanation could be given, were not taken into account for linear regression. Adsorbed fractions of Zn and Al in the presence of citrate also showed linear correlation with $r^2 = 0.85$ but any relation completely disappeared in the presence of EDTA (fig 1, A-4 and B-4). No correlation between Cu and Al adsorption was found when either citrate (A-5) or EDTA (B-5) was used as chelator.

Cation activity in the nutrient solution

Free metal activity of the nutrient cations in solution changed only marginally with the addition of 100 μM $AlCl_3$ (table 3). Equal concentrations of citrate and $AlCl_3$ (100 μM) lowered Al^{3+} activity ($\{Al^{3+}\}$) in the solution by 91% but also affected $\{Cu^{2+}\}$ (–33%). EDTA, as strong chelator, minimised not only Al activity but also reduced $\{Zn^{2+}\}$ in the solution by 95% and $\{Cu^{2+}\}$ by several orders of magnitude. Control experiments 3a1 and 3b1, with nutrient solution and chelators but without aluminium in the solution, confirmed the strong affinity of both chelators for Cu and of EDTA for Zn.

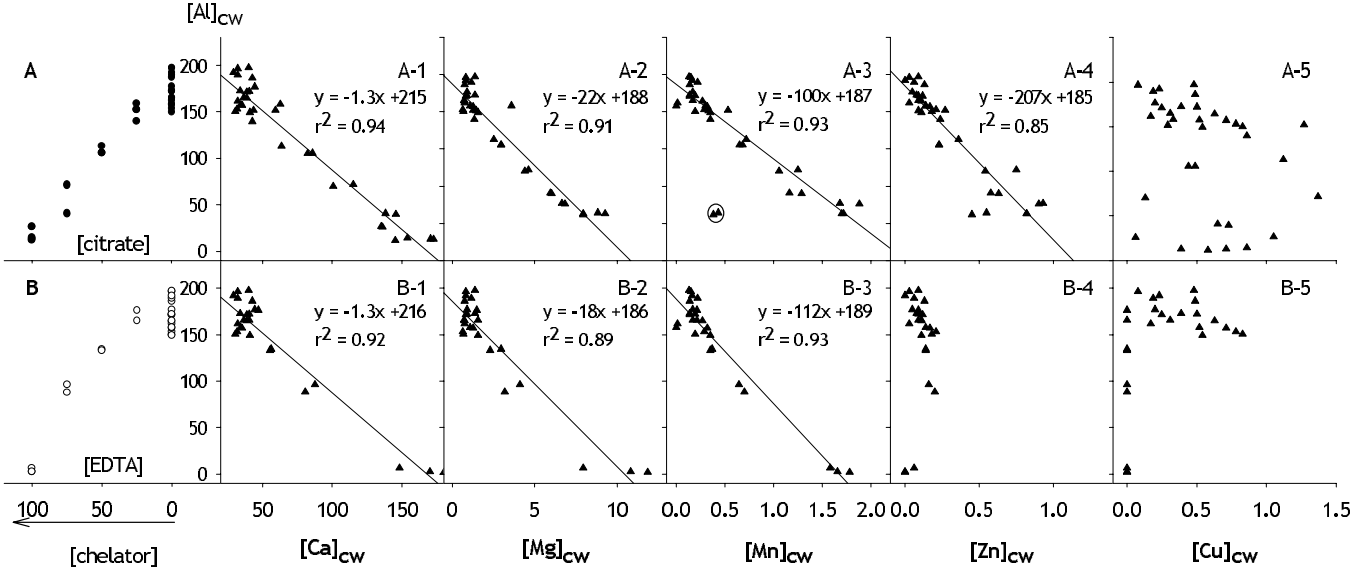

Figure 1: *Nutrient cation adsorption to isolated cell wall material as related to concurrent Al adsorption, in the presence of 100 µM $AlCl_3$. The first graphs on the left show the effect of increasing concentrations of citric acid (A) or EDTA (B) in the solution on Al adsorption to cell wall material. In the subsequent graphs 1-5, the effect of Al adsorption ($[Al]_{cw}$ on the Y-axis) on the adsorption of nutrient cations Ca (1), Mg (2), Mn (3), Zn (4) and Cu (5) (X-axes) is depicted. Linear regression and r^2 are indicated where relevant; outliers in A-3 (circled) are not taken into account for linear regression. All values for cation adsorption are expressed in µmol (g dry cell wall material (DCW))$^{-1}$; chelator concentration is given in µM.*

NS Cation	[cation]$_{NS}$ (µM)	Cation activity in nutrient solution (µM)						
				citrate (3a)				100 cit (-Al) (a1)
		NS (1)	100 Al (2)	25 (a2)	50 (a3)	75 (a4)	100 (a5)	
Ca	500	390	384	385	386	387	387	385
Mg	100	79	77	77	78	78	78	78
Zn	0.8	0.60	0.59	0.59	0.59	0.59	0.59	0.58
Mn	9.0	7.2	7.1	7.1	7.1	7.1	7.1	7.0
Cu	0.3	0.24	0.24	0.23	0.23	0.21	0.17	0.05
Al	0/100	0	46	34	23	13	4.3	0
				EDTA (3b)				100 EDTA (-Al) (b1)
		NS (1)	100 Al (2)	25 (b2)	50 (b3)	75 (b4)	100 (b5)	
Ca	500	390	384	384	385	385	386	376
Mg	100	79	77	77	77	77	78	78
Zn	0.8	0.60	0.59	0.54	0.46	0.33	0.03	3.8E-5
Mn	9.0	7.2	7.1	7.1	7.1	7.1	6.8	0.18
Cu	0.3	0.24	0.24	14E-3	4.8E-3	1.6E-3	7.5E-5	8.7E-8
Al	0/100	0	46	35	23	12	0.7	0

Table 3: *Cation activity in nutrient solution +/- 100 µM $AlCl_3$ and increasing concentrations of Al-chelator, as calculated with ECOSAT; solution pH = 4*

Taking the relative fraction in adsorption of a cation *x* to the cell wall and the relative cation activity in the solutions to calculate a fraction ratio, the cell wall showed a preferential adsorption for Cu >> Zn > Ca > Mn > Mg, except in the Al-EDTA treatment where Cu activity in the solution was reduced (table 4). Affinity for the trivalent Al was higher than for any of the divalent cations in all treatments.

$$\text{Fraction ratio} = \frac{\text{fraction of cation x in total cation adsorption}}{\text{fraction of cation x in total solution activity}}$$

Cation (x)	Fraction ratio			
	control (1)	+ $AlCl_3$ (2)	+ Al/citrate (3a5)	+ Al/EDTA (3b5)
Ca	1.7	0.3	1.4	1.7
Mg	0.5	0.04	0.4	0.5
Zn	5.3	0.6	4.6	2.74
Mn	0.7	0.1	0.7	1.0
Cu	16.0	5.9	13.4	0
Al		12.6	14.7	21.4

Table 4: *Fraction ratio: the ratio between the relative concentration of a cation in total adsorption and in total solution activity. At a fraction ratio > 1, a cation is preferentially adsorbed from the solution.*

Interaction of the cell wall with Fe^{3+} and PO_4^{3-}

In the acid desorption solutions concentrations of Fe and P were also measured, since these elements are known to co-accumulate with Al in the plant root. Average Fe^{3+} concentration was around 12 μmol $(g\ DCW)^{-1}$, without showing correlation with the presence of Al or Al-chelates (data not shown). P concentration in the desorption solution rose from 0 in the control treatment to 11 μmol $(g\ DCW)^{-1}$ in the Al treated samples. P is known to associate with Al^{3+} in plant roots. With chelation of the solution Al by citrate or EDTA, P accumulation is decreased but remains significantly above the control values (data not shown).

Discussion

The large fraction of adsorbed Ca on the cell wall material in the absence of Al is in concordance with the high Ca concentrations found in cell walls in living plants, where Ca plays an important role in cell wall and membrane stability (Rengel, 1992). The lower affinity of cell walls for Mg and Mn (Sentenac and Grignon, 1981; Tepfer and Taylor, 1981) and the low fraction ratios in the experiments described here also agree. Plant cell walls have a high affinity for micronutrient cations, especially for Cu and Zn (Kochian, 1991; Tepfer and Taylor, 1980), and relatively large amounts are adsorbed from the solution onto the cell wall surface. The high affinity of the cell walls pectic matrix for Al has been documented before (Ma *et al.*, 2001; Schmohl and Horst, 2000; Zhang and Taylor, 1990) and was also clearly visible in the competition with Ca for cell wall binding, described in chapter 2 of this thesis. It forms the basis for the extensive Al accumulation found in plant roots on acid mineral soils and it can again be seen in the high concentration of adsorbed Al to the isolated cell wall material in our experiments.

Al had a very strong direct effect on the adsorption of all cations in the solution. The reduction in amounts of Ca and Mg adsorbed to the cell wall (table 2) is in line with literature on Al effects on Ca and Mg concentrations in plant roots (Godbold and Jentschke, 1998; Jentschke *et al.*, 1991; Keltjens, 1995). A number of studies exist on interaction of trace elements with the root apoplast upon entering the root but to our knowledge there are no data on the competition for adsorption sites between aluminium and trace elements. The cations in the solution all compete for the same cell wall binding sites, which favour trivalent Al over the lower charged cations (table 4). The strong linear correlation between adsorbed Ca, Mg, Mn and Zn with $[Al]_{cw}$ confirms the competition for binding sites (fig. 1). $[Cu]_{cw}$ was less affected by Al than the other cations, as a result of the high preferential binding of Cu to cell walls or possibly of partial binding of Cu to other sites than the ones Al was competing for (Kochian, 1991; Parker *et al.*, 1998). Free ion activity of the nutrients in the solution containing 100 μM $AlCl_3$ did not change appreciably, when compared to that of the control treatment, and can therefore not be an explanation for decreased nutrient adsorption (table 3).

Complexation of the free aluminium in the solution by citrate or EDTA proved very effective for reducing free Al^{3+} activity in the solution and $[Al]_{cw}$: $\{Al^{3+}\}$ decreased to 9% and 1% of that in the $AlCl_3$ treatment and adsorption is lowered to 10% to 2%, with citrate and EDTA, respectively.

Ca and Mg adsorption remained at control level, in the presence of complexed Al. Mg was still significantly affected in treatment 3a4: as the least preferentially bound cation (table 4), Mg is more sensitive to low concentrations of free Al than the other nutrient cations. Zn adsorption also benefited from citrate, with cell wall concentrations staying at control level at citrate > 50 μM. The effect of EDTA on Zn adsorption can be explained by the reduction of the free ion activity in solution: $\{Zn^{2+}\}$ and $[Zn^{2+}]_{cw}$ are 5 and 2.5% of the control, respectively (table 3). Citrate in treatment 3a5 improved the Cu adsorption to the cell wall to a concentration, which was not significantly different from that in nutrient solution. Yet, a linear correlation between $[Al]_{cw}$ en $[Cu]_{cw}$, like the one for Zn or Mn, could not be found. Cu and citrate interact, as seen in the reduction in solution activity for Cu. $\{Cu^{2+}\}$ and the average $[Cu^{2+}]_{cw}$ are both reduced by one-third when treatments 1 and 3a5 are compared. A reduction in ion activity seems an obvious explanation but the results from 3a1, in which free Cu^{2+} activity is reduced by 80% but adsorption is similar to that in 1 and 3a5, contradict this. Both the fact that $[Al]_{cw}$ and $[Cu]_{cw}$ are influenced by a third factor (citrate) and the possible presence of other, Cu specific, binding sites, contributed to the lack of correlation in figure 1A-5. EDTA in the solution clearly reduced free Cu activity to such low concentrations, that no detectable adsorption on the cell wall took place (tables 2 and 3).

The results of treatments 3-a1 and -b1 and the fact that graphs A-1, -2 and -3 and B-1, -2 and -3 are identical show that citrate and EDTA did not interfere with the adsorption of Ca, Mg and Mn. Their cell wall binding was directly related to the concentration of $[Al]_{cw}$.

When the charge of the adsorbed cations is summed, the total charge on the cell wall amounted to 358 meq $(g\ DCW)^{-1}$ in the nutrient solution treatment, with Ca as the main contributor with 330 meq $(g\ DCW)^{-1}$. This value is close to the 375 meq $(g\ DCW)^{-1}$ for adsorbed Ca described in chapter 2, both as measured value and as predicted adsorption in the exchange model.

The increase in total adsorption sites in the presence of Al in the previous chapter is also visible here. In the experiments with Al in nutrient solution, 588 meq $(g\ DCW)^{-1}$ are occupied by nutrient cation and Al, which relates well to the 570 (measurement) to 600 (model prediction) meq $(g\ DCW)^{-1}$ in the Al/Ca experiments. With comparable amounts of Al and Ca adsorbed to the cell wall at pH 4 when $CaCl_2$ or nutrient solution is the background solution and assuming a similar available site number of 740 meq $(g\ DCW)^{-1}$ for the tomato roots, the model predictions from chapter 2 may very well apply to the nutrient solution system used here.

If Al indeed binds to other sites besides the carboxylic groups in pectin, which the cations compete for, then the fraction ratio for Al in table 4 should be read as a value for overall preferential binding to the cell wall and not specifically to the carboxylic groups. Nevertheless, accumulation of Al on the cell wall will influence nutrient cation binding and possibly nutrient uptake.

Taking the cell wall out of its context is a simplification but since it is the major cation exchanger in the apoplast, it can still give important information on processes in the root apoplast. Differences between results from whole plant experiments and *in vitro* experiments can be expected, with the higher accessibility of cell walls for cation adsorption when the root structure is opened up and cell membranes and cell contents are removed (Hart *et al.*, 1998). In a living root, the soil solution will have to penetrate into the apoplast matrix, with its variety of adsorption conditions.

The results from the experiments described here indicate the strong effect of Al on nutrient adsorption to the root cell wall. If cation adsorption is indeed a promotional step in nutrient uptake over the plasma membrane, then Al in the soil solution can reduce nutrient uptake by inhibiting this nutrient adsorption. Complexation of the free Al by ligands is an effective way to prevent the Al effect on nutrient adsorption, provided the ligand does not itself interfere with the nutrient cation adsorption.

4

Aluminium-induced differential decrease in nutrient uptake in tomato (*Lycopersicon esculentum* L.) and wheat (*Triticum aestivum* L.)

Abstract

Aluminium at phytotoxic concentrations decreases root growth and competes with nutrient cations for plant uptake. Of the nutrient cations, Ca and Mg have always received most attention and relatively little is known of the interaction between Al and micronutrients like Zn and Cu. Both Al and nutrient cations can bind to cell walls and membranes in the plant root and the competition for binding sites is thought to play an important role in the Al effect on nutrient uptake.

Tomato and wheat, which have different capacities to bind Al in their root cell walls, were chosen for the Al toxicity study described here. Seedlings were exposed to 100 μM $AlCl_3$ in nutrient solution for a period of 3 weeks and sampling at several moments during this period revealed differences in Al accumulation and plant response in growth and nutrient uptake. Plants were exposed in a similar set-up to Al with or without increasing concentrations of the Al chelator EDTA.

Al strongly decreased Cu and Zn uptake in both plant species. It also induced Mg deficiency but Ca concentration, which is usually affected in Al toxicity, did not become deficient within the 3 weeks of this experiment. Rapid Al accumulation in tomato roots caused an immediate reduction in relative uptake rate of all nutrients, whereas in wheat Al accumulation was slower and only uptake of Mg and the trace elements was affected from the start of Al exposure. Addition of EDTA could prevent Al inhibition of growth and nutrient uptake in tomato but wheat growth was severely reduced at the highest EDTA concentration.

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Submitted

Introduction

Aluminium forms the major problem in acid mineral soils in large parts of the world's arable land. A phytotoxic concentration of monomeric Al severely inhibits elongation of the plant root and plants grow poorly (Barceló *et al.*, 1996; Rengel and Robinson, 1989b). Inhibition of root elongation limits the possibility of a plant to explore the soil for nutrients and water. Besides being spatially limited, the uptake of nutrients is directly influenced by the presence of the toxic metal. Al accumulates in the apoplast of the cortex and tips of newly formed roots. There it adsorbs to negative binding sites on the root cell wall and the plasma membrane, taking the place of nutrient cations and protons (Archambault *et al.*, 1996; Jentschke *et al.*, 1991). For several nutrient cations a positive correlation between apoplast binding and uptake into the symplasm has been established, although the role of cell wall adsorption in this is not always understood (Keltjens, 1995; Sattelmacher, 2001). Adsorption of Al instead of nutrient cations may interfere in this uptake pathway by preventing the nutrient cell wall adsorption. Al will bind to the apoplasmic side of the plasma membrane, this way altering the membrane surface potential and blocking ion-specific channels (Gassmann and Schroeder, 1994; Kinraide *et al.*, 1992; Rengel, 1992). A further possibility for Al interference in nutrient uptake exists in gramineae, where it can inhibit the production and/or exudation of the metal-binding phytosiderophores, thus decreasing possibilities for the plant to take up Fe and Zn (Chang *et al.*, 1998; Hart *et al.*, 1998). Although Al clearly affects many processes in the root apoplast and symplast, its precise toxic effects on growth and nutrient uptake, as well as the mechanism for uptake of Al into the cytoplasm, are yet to be explained.

Changes in nutrient uptake, as induced by Al, will of course affect the plant. As long as the relative nutrient uptake rate in a plant is equal to the plant's relative growth rate, the overall concentration of that nutrient in the plant will remain constant (Ingestad and Ågren, 1992). A decrease in nutrient uptake will initially lead to a lower concentration in the plant and, when this concentration drops below a critical value, to altered dry matter production.

Except for being a consequence of nutrient deficiency, reduced growth has at the same time the effect of limiting the deficiency by diluting the available pool of nutrients less.

Is then the effect of Al on plant growth purely a matter of nutrient deficiency? Not exclusively. Al inhibits root elongation long before nutrient concentrations become deficient and this aspect of the growth inhibition involves deregulation of apoplasmic as well as cellular processes (Kenjebaeva *et al.*, 2001; Ryan *et al.*, 1992). The decrease in dry matter production which occurs later can be a result of a nutrient deficiency, yet it may also be a direct toxic effect of Al on growth, e.g. through changing plant levels of cytokinin and ethylene (Massot *et al.*, 2002).

In studying the relationship between Al and nutrient uptake, much emphasis has been put on the macronutrients. Ca and Mg are probably the best-studied nutrients in this respect. Their concentration in plant shoots generally decreased when plants were exposed to Al but this decrease did not immediately lead to a reduction in growth (Godbold and Jentschke, 1998; Göransson and Eldhuset, 1995). The initial toxic effect of Al concerning these nutrients more likely lies in disturbing the Ca homeostasis in the cell wall and the cytoplasm and in interfering with Mg-sensitive channels (Silva *et al.*, 2001b; Zhang and Rengel, 1999). The response of K to high Al varies from increase to decrease in shoot K concentration (Camacho and Ramirez, 1995; Rengel and Robinson, 1989b). P accumulates in the root, probably as an Al-phosphate complex in the apoplast or the vacuole, and transport to the shoot is either decreased or not affected (Macklon *et al.*, 1996; Rout, 2001). Studies on the effect of Al on micronutrient uptake are more scarce and although in general uptake of cations is decreased, Al at low concentration on occasion has had a promoting effect on cation uptake (Baligar *et al.*, 1993; Simon *et al.*, 1994). Often inconsistencies in Al effects may be reduced to the wide variety in plant species and experimental designs that have been used. Results will vary with the choice of plant species and age, duration of the experiment, Al concentration, pH and ionic strength in the solution (Lazof and Holland, 1999; Wheeler, 1994). Between species the difference in Al tolerance can be extensive, a fact which is often used in studying Al toxicity. Tolerance to Al can be realised by root exudation of various chelators for complexation of Al,

excluding Al from the root or sequestering it in a complex in designated plant parts (Keltjens, 1997; Ma *et al.*, 1997a; Piñeros *et al.*, 2002). It is generally accepted that Al complexation is an effective mechanism for a plant to decrease Al toxicity by decreasing its capacity to bind to negative binding sites in the root apoplast (Blamey *et al.*, 1997; Kidd *et al.*, 2001).

To study the development of Al toxic effects on plant growth and nutrient uptake in time, with specific emphasis on the micronutrients, seedlings of tomato and wheat were grown for a period of 3 weeks on 100 μM $AlCl_3$ in a nutrient solution. Plant samples were taken at eight different moments and analysed for growth and nutrient concentration. To compare the effect of Al in a complexed form with that of free monomeric Al, a second experiment was performed, similar in time and set-up but with the addition of increasing concentrations of Na_2-EDTA as Al-chelator. Since Al affects both growth and nutrient uptake, thus influencing the nutrient concentration of the plant from several angles, both tissue nutrient concentration and content were used to analyse the impact of Al.

Plant species were selected as being relatively Al sensitive and to represent the two plant subclasses di- and mono-cotyledons. One of the differences between these two subclasses is the composition of the root cell wall: the much higher pectin content in dicots creates a larger capacity to bind cations, both nutrient cations and Al. Schmohl and Horst (2000) have shown that an increased pectin content in maize was related to an increase not only in Al accumulation in the root but also in sensitivity to Al.

Materials and Methods

Plant material

Seeds of tomato (*Lycopersicon esculentum* L. cv. Moneymaker) and summer wheat (*Triticum aestivum* L. cv. Minaret) were germinated for 10 (tomato) or 7 days (wheat) in moist quartz sand, at 20°C. Subsequently, seedlings were transferred to 50 L containers with a standard nutrient solution, in climate controlled rooms at a regime of 20°C, 16/8 hours light/dark (light intensity was 70 W m^{-2}) and 80% relative air humidity. The standard nutrient solution consisted of (in mM): Ca (1), K (2), Mg (0.25), N (4, as NO_3^-), P (0.075) and

S (0.25), and trace elements (in μM): Fe (95), B (46), Zn (0.8), Mn (9), Cu (0.3) and Mo (0.1). Solution pH was kept at pH 5.5 by of automatic titration. The pH was lowered to 4 after 6 days of pre-culture, one day prior to the start of the experiments; nutrient solutions were refreshed twice a week.

Experimental set-up

Aluminium effects in time on growth and nutrient uptake (exp. 1)

For the first experiment, sixty seedlings of tomato and wheat were grown on 50 L containers with standard nutrient solution at pH 4, with the addition of 0 or 100 μM $AlCl_3$ for the different treatments. Per treatment and per plant species, 4 containers were used. Solutions were refreshed twice a week and pH was kept at 4.0. Al^{3+} activity in the nutrient solution, at this pH, is calculated to be 22 μM. Speciation calculations were performed with the use of ECOSAT (Equilibrium Calculation Of Speciation And Transport (Keizer *et al.* (1992)).

Plant samples were taken from each container before the start of the experiment (day 0) and at 7 moments during the following 3 weeks, with increasing time intervals (table 1). At each harvest 2 sets of plants from different containers with a similar treatment were combined to a total sample of *n* plants, for growth and chemical analysis. This resulted in duplicate data sets for every treatment.

The effect of complexation of Al by EDTA on growth and nutrient uptake (exp. 2)

Growth conditions in the second experiment were similar to those in the first one, with 50 tomato or 70 wheat seedlings per container. A total of 6 treatments were applied, on single containers: a control treatment of standard nutrient solution, addition of 100 μM $AlCl_3$ and of 100 μM $AlCl_3$ plus 25, 50, 75 or 100 μM sodium-ethylenediamine tetraacetic acid or Na_2-EDTA. The addition of increasing concentrations of EDTA decreased Al^{3+} activity in the nutrient solution from its initial 22 μM (0 EDTA) to 15, 9, 4 and 0.3 μM (with 25, 50, 75, 100 μM EDTA), respectively (ECOSAT).

Samples were taken before the start of treatments (day 0), at day 9 (wheat) and 10 (tomato), and a final harvest at day 20 and 21, respectively; table 1). Samples at day 0 were collected from all 6 containers and divided over 2 sets of *n* plants. At the other harvests, two sets of *n* plants each were harvested per treatment, per plant species.

Exp 1: Al effect in time				
	tomato		wheat	
	0 Al	100 Al	0 Al	100 Al
day	*n*			
0	16	16	16	16
1	16	16	16	16
2	16	16	16	16
3			16	16
4	16	16		
7	16	10		
8			12	12
10	10	12	10	10
15	8	12	9	10
20			9	10
21	6	12		
Exp 2: the effect of Al-EDTA				
	tomato		wheat	
	0 Al	100 µM Al +/- EDTA	0 Al	100 µM Al +/- EDTA
day	*n*			
0	75	75	22	22
9			15	13
10	7	7		
20			12	14
21	7	7		

Table 1: *Sampling schedule for Al effect on plant growth and nutrientuptake, over a 3 week period; n indicates the number of plants per treatment sample.*

Plant analysis

Growth analysis: plant samples were separated into shoot and root parts and weighed. The roots were rinsed for 60 sec in demineralized water and blotted dry. Portions of 500-700 mg root material were taken for measuring root length, using the method of Newman (1966). The shoot and root material was oven dried, overnight at 70°C, and subsequently weighed. Dry weight and root length are expressed as a percentage of the values found in the control treatment (0 Al). For experiment 1, the natural logarithm of total plant dry weight is plotted against time to show the relative growth rate (RGR) in the control and the Al treatments.

Chemical analysis: samples of the dry plant material were analysed for chemical composition after a microwave HF-HNO_3-H_2O_2 digestion (Novozamsky *et al.*, 1996). Total concentrations of Al, Ca, Mg, K, Zn, Cu, Mn, P and Fe in the digest were measured with inductively coupled plasma-atomic emission spectrometry (ICP-AES).

Al and nutrient concentrations in shoot or root are expressed in μmol $(g\ DM)^{-1}$. Nutrient content per total plant was calculated for every measured element and the ln of each content was plotted against time. The slope of the resulting graphs indicate the relative uptake rate (RUR) per nutrient, per plant.

Results

Effects of Al^{3+} on growth and nutrient uptake in tomato and wheat seedlings in time

All growth parameters measured (shoot and root dry weight and root length) were strongly inhibited by aluminium in both plant species and were at the final harvest around 5% (tomato) and 20% (wheat) of the control plants (fig. 1). Growth in tomato seedlings was affected from day 1, whereas in wheat, root elongation was inhibited immediately but dry matter production was not affected by Al until after 3 days.

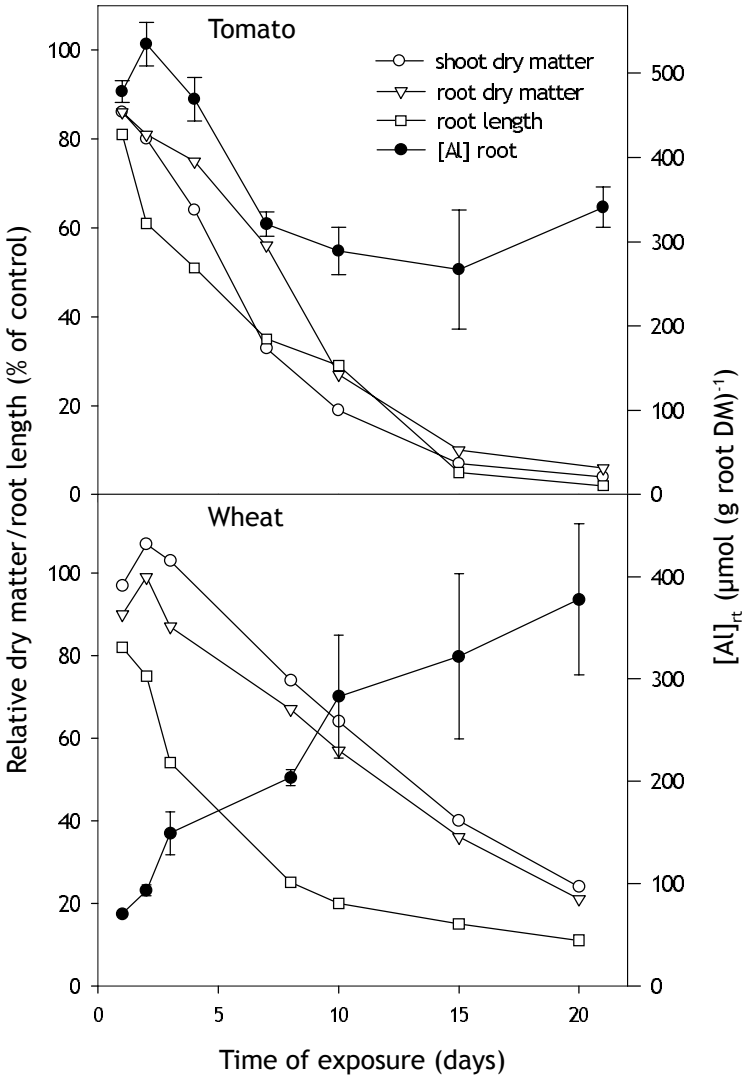

Figure 1: *Relative total shoot and root dry matter production and relative root length in tomato and wheat, during 3 weeks of exposure to 100 µM $AlCl_3$ in nutrient solution, as % of the control (left axis). Values for the Al concentration in the root tissue are the averages of two samples, with their respective standard deviations (right axis).*

Setting out the ln of total plant dry matter against time gave the linear relationships of exponential growth for both control and Al treated plants (fig 2). Al concentration in the root at the final harvest was around 350 μmol (g DW)$^{-1}$ in both tomato and wheat but the progress of accumulation differed noticeably between the species (fig. 1). Tomato had a high root Al concentration from day 1, while Al concentration in the wheat root gradually increased over time. Only 1-3% of total Al was transported to the shoots of both species, causing shoot concentrations of 1-3 μmol g^{-1}. Tomato relative growth rate (RGR) was moderately reduced in the first 4 days, compared to the control but decreased dramatically from 0.23 to 0.10 after day 4 (fig. 2A). Dry matter production in the wheat plants did not respond to Al in the first week of the experiment and only after day 8 did the RGR decrease from 0.20 to 0.10 (fig. 2B). Root elongation rates of both species were decreased to a lower constant value from the moment of Al addition (data not shown).

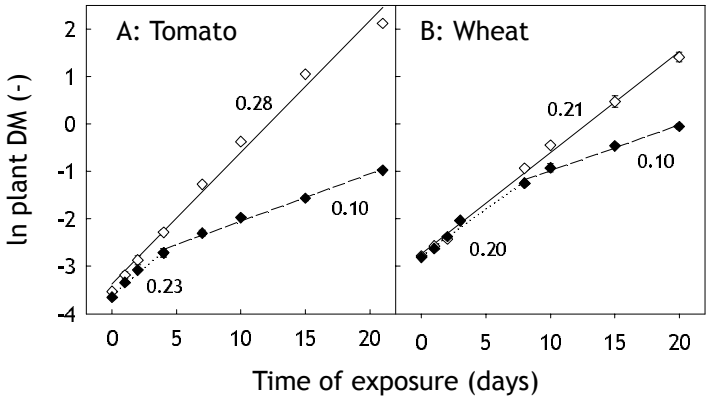

Figure 2: *Total dry matter of tomato and wheat plants, during a 3 week period of exposure to 0 (open symbols) or 100 μM $AlCl_3$ (closed symbols) in nutrient solution. Plant dry matter is given as the natural logarithm of the weight and is therefore dimensionless. Numbers in the graphs indicate the relative growth rate.*

Relative nutrient uptake rates (RUR) were constant in control plants of both species and were generally closely related to the RGR (fig. 3A and -B). Al addition decreased nutrient uptake in the tomato plants immediately, with RUR values for most nutrients at 30 to 50% of the control (fig 3A). Uptake of Cu and Mn was close to zero in the first week but started again after day 7. In Al-exposed wheat, the response of nutrient uptake to Al varied (fig 3B): the relative uptake rates for Mg, Zn and Mn severely decreased from the start of Al exposure, whereas Al limited uptake of Ca, K and P only to a lesser extent and not until after day 3. Cu uptake in wheat plants stopped after 2 days and did not resume during the remainder of the experiment.

Besides a strong decrease in nutrient uptake, also the shoot/root partitioning of some of the nutrients was altered. Had the tomato shoot at the final harvest received 80% of total Cu in the control plants, in Al affected plants this had decreased to 40%, while the shoot's share in total dry matter only decreased from 88 in the control to 79% in Al-treated plants. P also accumulated in these roots, as 52% of total P remained in the root compared to 17% in the roots of the control. In wheat, Al had a similar though milder effect on P partitioning. Relatively speaking, these plants retained much more Fe in their roots (79% of total Fe in stead of 33%) but they destined 50% more Mn to the shoot parts.

The combination of decreased nutrient uptake and inhibited growth lead to changes in plant tissue concentrations. Arrows in figure 3 indicate the moments in the experiment when, in Al-treated plants, the shoot concentration of a given nutrient became deficient (reference values from de Kreij *et al.* (1992) and Reuter *et al.* (1997)).

In the control tomato plants, relative growth and relative nutrient uptake kept pace at a rate of around 0.28 day^{-1}. Cu uptake was an exception in that it was low and resulted in Cu deficiency in the shoots from day 10 onwards, possibly as a result of a too marginal solution Cu concentration. Although exponential growth apparently was not affected by it, the relative uptake rate of Cu may have been higher in these plants under more optimal conditions and may have conformed more to the general nutrient uptake rate of 0.28 day^{-1}.

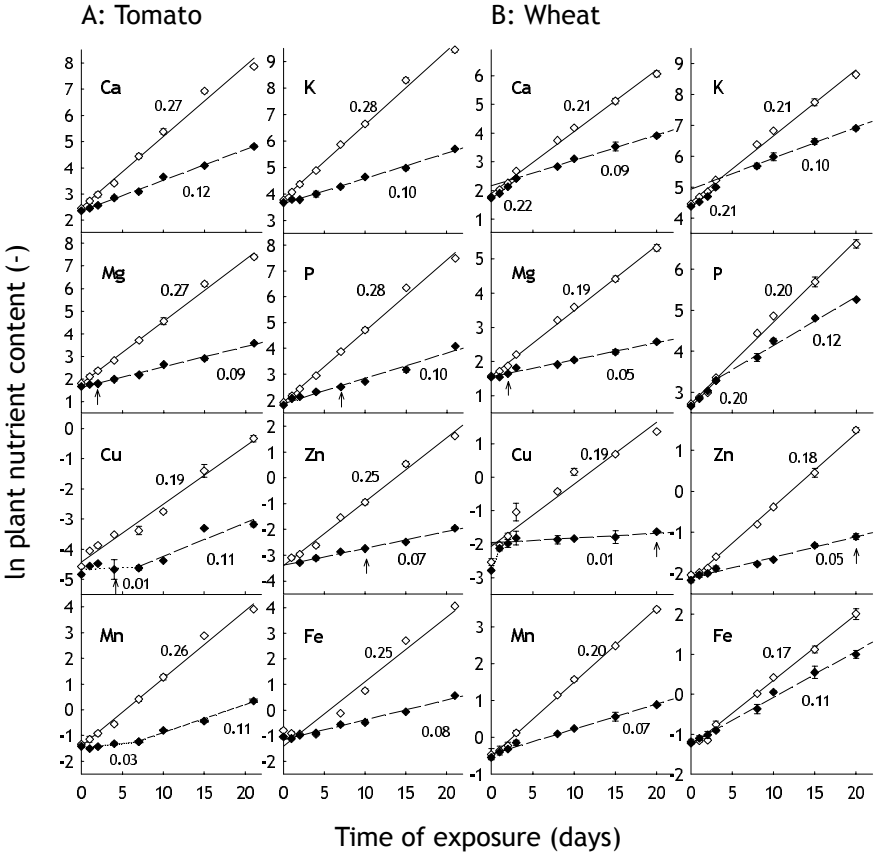

Figure 3: *Nutrient content in tomato (A) and wheat (B), during a three week period of exposure to 0 or 100 µM $AlCl_3$ in nutrient solution. Open and closed symbols represent –Al and + Al treatment, respectively. Numbers in the graphs indicate the relative uptake rate (RUR) of the nutrient. An arrow indicates if and when a nutrient concentration in the shoot became deficient.*

The effect of addition of EDTA on Al toxicity

Again, 3 weeks exposure to 100 μM Al reduced dry matter production and root elongation in both plant species to only a fraction of the growth in control plants (fig. 4). Simultaneous addition of EDTA and Al decreased the effects of Al with increasing EDTA concentration (fig. 4 and 5A). In tomato plants, dry matter production was close to control level with the 75 μM EDTA but root length was strongly inhibited, until EDTA concentration reached 100 μM. Wheat growth also markedly improved with the addition of EDTA but never reached the control values; especially root length remained inhibited in all treatments (fig. 4 and 5B).

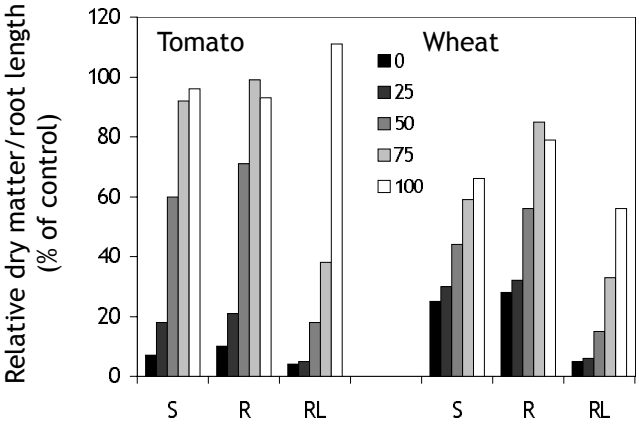

Figure 4: *Relative shoot (S) and root (R) dry matter and root length (RL) in tomato and wheat, after 3 weeks growth in nutrient solution with 100 μM Al Cl_3 and 0-25-50-75-100 μM EDTA, respectively. Plant dry matter and root length in the absence of Al is taken as reference (= 100%).*

Comparable to what was found in experiment 1, 96 to 98% of total Al in the plants was retained by the root tissues in the 100 μM Al treatments. Total Al content in tomato plants increased with addition of 25 or 50 μM EDTA but decreased at the highest EDTA treatments (table 2, Al_{sht} and Al_{rt}). In the presence of EDTA, slightly more Al was transported to the tomato shoot. This is in contrast with Al accumulation in wheat plants, in which Al accumulation decreased with increasing EDTA concentration and shoot concentrations did not rise above a background level at any Al treatment.

Figure 5: *Tomato (A) and wheat (B) plants after 3 weeks growth on nutrient solution (C), with addition of 100 μM $AlCl_3$ and 0, 25, 50, 75 or 100 μM Na_2-EDTA (1-5, respectively).*

Like in experiment 1, total uptake of all measured nutrients was strongly reduced in Al treated plants. Total nutrient contents in table 2 show that this effect had been increasingly counter-acted by raising the EDTA concentration in the nutrient solution. Nutrient content in tomato plants under treatment with equal concentrations of Al and EDTA was around the control level, except for Fe, Zn and Cu. In wheat, only Mg and Mn were taken up in amounts equal to the control; of the other nutrients, especially Zn and Cu were limited in uptake at the highest EDTA concentration.

treatment Al/EDTA (µM)	Al (µmol plant^{-1})		Nutrient content of total plant (in % of the control)							
tomato	Al_{sht}	Al_{rt}	Ca	Mg	P	K	Fe	Zn	Mn	Cu
100/0	1	28	6	4	6	3	8	3	4	8
100/25	3	38	13	9	14	8	11	7	9	5
100/50	11	36	56	35	59	43	26	20	45	13
100/75	11	17	115	69	96	87	38	43	83	28
100/100	5	4	122	89	98	94	50	70	100	27
wheat										
100/0	0.7	49	14	9	28	19	85	8	10	6
100/25	0.8	21	18	11	29	23	53	11	13	4
100/50	0.7	14	36	21	49	38	53	25	28	9
100/75	b.d.	11	55	49	73	58	74	48	62	22
100/100	b.d.	1	74	102	76	57	75	38	112	14

b.d.= below detection limit of ICP-AES

Table 2: *Al accumulation in the shoot (Al_{sht}) and root (Al_{rt}) and nutrient content of whole tomato and wheat plants, which were treated for 3 weeks with 100 µM $AlCl_3$ or 100 µM $AlCl_3$ and increasing concentrations of Na_2-EDTA. Values for nutrient content are given as percentage of the control treatment T1 (nutrient solution).*

Figure 6 shows the relative uptake of Mg and Zn in both species, as examples of uptake of macro- and micro-elements, with the 0 and 100 µM Al lines from experiment 1 as reference (dashed lines). Ca, K, P and Mn gave results very similar to Mg uptake, with adequate nutrient uptake at the highest EDTA levels. EDTA caused only a minor increase in Cu uptake at 50 µM EDTA and higher (table 2).

Shoot tissue concentrations found in both species at the final harvest as the result of uptake and growth are given in table 3. Critical nutrient concentration values indicate the minimal requirement for growth. 100 µM Al caused Mg, P, K, Zn and Cu deficiency in tomato. This effect was prevented for all nutrients, except Cu, by 100 µM EDTA in the solution. In wheat, only Mg and Cu became deficient in the Al treatment and no deficiency was found in the shoots at EDTA > 50 µM. Cu concentrations in the wheat shoots treated with 100/75 and 100/100 µM Al/EDTA were increased due to an increase in shoot/root partitioning of total Cu. The Cu concentration in the roots of these plants was limited to 5% of that of the control, possibly leading to a Cu deficiency in the root.

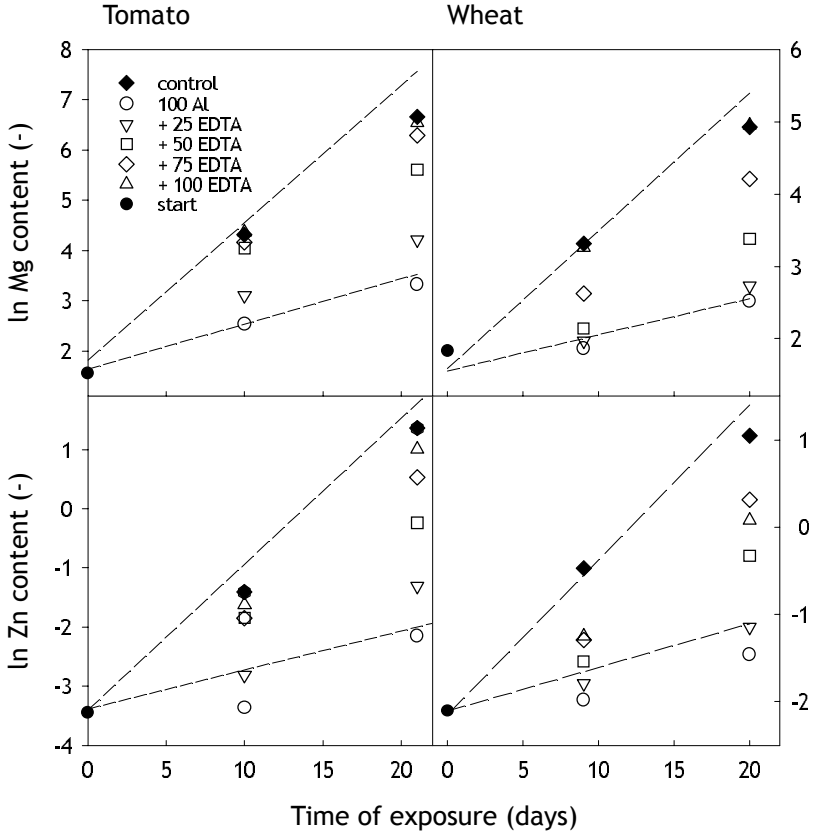

Figure 6: *Relative uptake of Mg and Zn in tomato and wheat, in the presence of 100 µM $AlCl_3$, with addition of increasing concentrations of Na_2-EDTA (see legend for explanation of the symbols; corresponding symbols relate to equal treatments). The lines in each graph are the regression lines from the previous experiment, in which plants were treated with 0 or 100 µM Al Cl_3 in nutrient solution (top and bottom line, respectively).*

treatment Al/EDTA (µM)	$[Al]_{rt}$ (µmol g^{-1})	Shoot concentration (µmol (g DM)$^{-1}$)								
tomato		Al	Ca	Mg	P	K	Fe	Zn	Mn	Cu
0/0	1.2	0.6	410	186	148	1359	5.1	0.79	5.2	0.10
100/0	521	4.3	331	89	65	563	2.7	0.30	2.9	b.d.
100/25	318	3.8	293	83	70	531	1.6	0.23	2.5	b.d.
100/50	91	4.6	381	106	118	893	1.8	0.23	3.8	b.d.
100/75	30	2.9	520	140	147	1237	2.3	0.34	4.7	b.d.
100/100	7.5	1.2	525	177	153	1285	3.2	0.52	4.8	b.d.
critical concentration:			200	150	100	600	1.1	0.47	0.9	0.08
wheat										
0/0	b.d.	b.d.	112	51	177	1664	1.6	1.08	4.1	0.18
100/0	286	1.3	63	17	151	1307	1.9	0.29	1.9	0.05
100/25	110	1.1	65	17	150	1314	2.1	0.33	2.1	b.d.
100/50	40	0.7	89	23	191	1381	1.9	0.55	3.4	0.08
100/75	21	b.d.	99	41	206	1525	2.1	0.77	4.8	0.15
100/100	2	b.d.	125	85	195	1390	2.0	0.57	7.4	0.11
critical concentration:			38	21	81	1000	0.4	0.23	0.2	0.08

b.d.= below detection limit of ICP-AES

Table 3: *Al accumulation in the roots ($[Al]_{rt}$) and Al and nutrient concentrations in the shoots of tomato and wheat plants, which were treated for 3 weeks with 0, 100 µM $AlCl_3$ or 100 µM $AlCl_3$ and increasing concentrations of Na_2-EDTA. Critical nutrient concentrations in shoot tissues, considered as minimal requirement for growth, are given in the last row of each block. Shoot nutrient concentrations which decreased below these critical levels are indicated by grey shading (values from de Kreij et al. (1992) and Reuter et al. (1997)).*

Discussion

Al uptake and the effect it had on nutrient uptake and growth in tomato distinguished itself from that in wheat in several respects. The rapid Al accumulation in the tomato roots in experiment 1 and the difference in concentration between tomato and wheat roots in experiment 2, can both be a consequence of a higher pectin content in the root of a dicot, like tomato. Wheat roots accumulated Al more slowly but still reached a relatively high Al concentration at the final harvest. Wheat takes up lower amounts of cations per unit NO_3^- than tomato does and, in order to fulfil the requirements for charge balance, relatively more OH^- will have to be exuded (Marschner, 1995; chapter 5 of this thesis). The resulting increase in apoplast pH may be responsible for a progressive precipitation of Al-hydroxides in the root.

High root accumulation is expected to increase Al uptake into the cytoplasm and transport to the plant shoot. Tomato roots with their immediate high Al accumulation would be expected to take up and transport more Al to the shoot than the slowly accumulating wheat plants but this can not be concluded from experiment 1. The response in uptake may be too slow to be made visible within the time span of this experiment, or the measurements with the ICP-AES may not have been sensitive enough to detect significant differences. Al concentrations in tomato shoots in the Al treatment of the second experiment were higher at higher root Al concentration, compared to the wheat plants, but these data are not sufficient for a definite correlation. The difference in Al accumulation in tomato and wheat can account for the difference in growth response. As long as the Al concentration in wheat roots is low, dry matter production can continue as normal, while the high Al concentration in tomato inhibits growth immediately. Root elongation, the most Al-sensitive of growth characteristics, is inhibited even at the lowest Al concentration.

EDTA in the nutrient solution decreased the Al accumulation in the plants and generally increased growth. At higher concentrations, EDTA minimises the activity of monomeric Al in the solution and therewith decreases the possibility for Al to adsorb and accumulate in the root cell walls. Yet, the higher Al content in tomato shoots in treatments with 100/50 and

100/75 μM Al/EDTA indicates that EDTA somehow seems to facilitate Al uptake and transport to the shoot, possibly as an EDTA-complex. On the other hand, at the highest EDTA concentration, when this effect would be expected to be maximal, shoot accumulation of Al is limited. Root accumulation in that treatment is also limited, indicating that cell wall adsorption may still play a mediating role in Al uptake, even in the presence of EDTA.

Al inhibited general nutrient uptake in the tomato roots in experiment 1 and, although uptake of most nutrients remained exponential, their relative uptake rate was only approximately one-third of that in the control. The effect of Al on total plant uptake was most dramatic for Cu and Mn, which almost came to a complete standstill. Although total Cu uptake started again after day 7, shoot concentration remained deficient as a result of inhibited shoot-root translocation. Such Cu retention has been reported before for an Al sensitive tomato cultivar and in tomato, grown at a higher rhizosphere pH (Chaignon *et al.*, 2002; Simon *et al.*, 1994). The re-start of uptake could be a result of the decrease in Al concentration in the tomato root from 500 to around 300 μmol $(g\ DM)^{-1}$, indicating a possible threshold value for inhibition of Cu and Mn uptake in that range. Like the dry matter production, Ca, K and P relative uptake rates were not affected in the first three days but subsequently switched to a lower rate, comparable to the RGR. Mg, Cu, P and Zn concentrations in tomato shoots became successively deficient. Shoot P deficiency was aggravated by accumulation in the root, a well-known feature in Al phyto-toxicity.

In wheat, uptake of Mg and the micronutrients was clearly more sensitive to Al than uptake of the macro elements and RURs were decreased at low Al concentrations in the root. Wheat is known to be sensitive to Cu deficiency (Marschner, 1995) and Al caused complete inhibition of Cu uptake. Only the Mg concentration in the shoot was deficient from day 2 onwards but growth was apparently not immediately hampered by it. It may, however, be in some way be linked to the root elongation. The only marked difference in nutritional status of the tomato plants between treatments with 100/75 and 100/100 μM Al/EDTA of experiment 2 is the fact that in the latter, Mg is no longer deficient, a change which coincides with the root elongation increasing to control level.

EDTA proved to be effective in reducing Al effects on uptake of most nutrients in both plant species but created an additional pressure on plant uptake of Cu and Zn. In tomato already at 50 μM EDTA overall nutrient uptake had increased, compared to the Al treatment without EDTA; growth was substantially increased and most macro-nutrient concentrations were no longer deficient. The increase of P and K uptake apparently formed the crucial improvement, while at the same time the extremely low Zn and Cu concentrations in the shoots did not inhibit growth. The high affinity of EDTA for Zn and Cu causes the solution concentrations of free metal ions to decrease sharply, impeding uptake (Laurie *et al.*, 1991).

Nutrient uptake and growth in wheat was still inhibited when Al activity was decreased to 0.3 μM with the highest concentration of EDTA. Wheat nutrient concentrations in the shoots were no longer deficient but this may have been more a result of a still limited growth than of improved uptake, since total uptake for most nutrients was still too low to support full growth. Additionally, the root concentrations of some nutrients may be limited but there is unfortunately little information on required root concentration. Zn uptake is severely hampered by both Al and by EDTA, though with the relatively low Zn demand in wheat, shoot concentrations are never below deficiency level. The relatively high Cu concentration in the wheat shoot in treatments with Al and 75 or 100 μM EDTA was mainly a result of a strong increase in Cu transport to the shoot, since total Cu uptake is very limited. The low Cu concentration and the sensitivity of wheat to Cu deficiency, possibly in combination with a higher sensitivity to long term exposure to low Al concentration, is considered to be the cause of stagnant growth in the wheat plants.

A decrease in Ca, Mg and P concentrations is generally considered to be the main toxic effect of Al on plant nutrition. The experiments presented here show a more complex image of Al toxicity by taking the micronutrients into account. Both plant species suffered from Mg deficiency, as expected, but a too low P concentration was only a problem in the tomato plants, and not in wheat. Neither plant species became Ca deficient, although uptake was reduced. More importantly, Al strongly affected the uptake and distribution of Cu and Zn in both plant species and this fact may provide new entries into unravelling the precise target of Al toxicity.

Acknowledgement

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5

Al specific inhibition of nutrient uptake in tomato and wheat; the effect of pH and the Al-chelator EDTA

Abstract

Plants growing on acid soils are often limited in growth and nutrient uptake as a result of toxic concentrations of Al^{3+} and the low pH in the soil solution. In most cases it is very difficult to distinguish whether effects are caused by either Al or low pH, since Al toxicity generally only occurs at pH < 5. Yet, the distinction is important for a full understanding of the phytotoxic effects of Al. To evaluate the relative importance of Al and proton activity in the solution on plant growth and nutrient uptake, seedlings of tomato and wheat were exposed to Al and different solution pH conditions in a complete factorial experimental set-up. Plants were grown on nutrient solutions with two different pH levels and Al present as 0 or 100 μM $AlCl_3$ or combined with the chelator Na_2-EDTA. In a second experiment, seedlings were grown on nutrient solutions with 0 or 50 μM $AlCl_3$ and with NO_3, NH_4 or a mix of both as N source, to create different pH levels in the plant root apoplast.

Plant growth generally responded strongly to Al and only little to a lower pH or NH_4 in the solution. Growth improvement by chelating Al was very limited. Al induced a specific decrease in Cu and Zn uptake and translocation in wheat, which was not affected by relatively small pH changes. However, the Al-induced decrease could be (partially) prevented with NH_4-nutrition, an effect, which is presumably caused by apoplast acidification as a result of NH_4-assimilation. The response in Mg concentration in wheat shoots was most likely pH independent and Al specific, whereas Ca and K concentrations did respond to the pH but only in the presence of Al. Nutrient uptake in tomato was generally sensitive to both low pH and Al. The use of an Al-chelator, like Na_2-EDTA, was effective in reducing Al-induced effects on nutrient uptake but could interfere itself with micronutrient uptake.

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To be submitted

Introduction

Aluminium toxicity in plants is visible in a rapid inhibition of root elongation, followed by a decrease in overall growth (Barceló *et al.*, 1996). At phytotoxic Al concentrations, also the plant nutrient uptake decreases, initially through Al interference with root nutrient uptake and additionally, in a later stage of growth, because of the smaller root system. Al accumulates in the plant root apoplast, especially at the root tips, where it binds to the cell wall and to the outside of the plasma membrane (Blamey, 2001; Godbold *et al.*, 1995; Högberg and Jensén, 1994). There it interferes with membrane and cell wall composition and functioning (Tabuchi and Matsumoto, 2001; Yamamoto *et al.,* 2001; Zhang *et al.*, 1996).

The uptake of Al and its effect on nutrient uptake are not fully understood. Interference with nutrient uptake is partly based on cation competition at apoplast binding sites, which have a higher affinity for the trivalent Al than for di- and monovalent nutrients like Mg and K (Rengel, 1992; Richter and Dainty, 1989). Other Al effects are more specific and involve direct inhibition of Ca and K ion-channels or plasma membrane H^+-ATPases (Ahn *et al.*, 2002; Ding *et al.*, 1993; Liu and Luan, 2001; Piñeros and Kochian, 2001; Rengel, 1992). Al can also cause callose formation, which was shown to block plasmodesmata, thus affecting cell-to-cell transport of water, nutrients and signalling molecules (Sivaguru *et al.*, 2000). On the plant level, Al causes a decrease in Ca and Mg concentration (Horst, 1995; Kidd and Proctor, 2000; Zsoldos *et al.,* 2000), which can be prevented by providing the plant with high Ca or Mg concentrations (Kinraide *et al.*, 1992; Silva *et al.*, 2001a; Tan *et al.*, 1992a). Al effects on other nutrients, like the trace elements Cu and Zn, are less well documented and interactions with Al toxicity are not fully known.

Al phytotoxicity is in many ways connected to the pH of the rhizosphere. In the first place is Al toxicity a problem of acid soils, where increasing proton concentrations replace Al at the surface of soil particles, leading to toxic concentrations of free Al^{3+} in the soil solution (Gahoonia, 1993; Göttlein *et al.,* 1999). The local proton concentration in the root apoplast will on the one hand determine the activity of Al^{3+} and nutrient cations in their competition for apoplast binding sites. On the other hand,

protons are also strong competitors for these same sites (Calba *et al.,* 1999; Grauer and Horst, 1992; Kinraide, 1991; chapter 2 of this thesis). The apoplast pH is the resultant of the activity of H^+-ATPases in the plasma membrane and the rhizosphere pH. Proton extrusion from the cells plays an important role in maintaining the negative membrane potential and the cytoplasmic pH, in cation and P_i uptake and in Fe(III) reduction (Thibaud *et al.*, 1994; Toulon *et al.,* 1992). It is also involved in growth by loosening the cell wall for cell elongation and is found at initiation sites for root hair formation (Bibikova *et al.,* 1998; Peters and Felle, 1999). By regulating the H^+ extrusion, a plant can alter its apoplast and rhizosphere pH to some extent, thus creating a more constant environment (Felle, 1998). However, a too low ambient pH will decrease the efficiency of the proton pumps (Marschner, 1995). The plant will suffer from proton toxicity, causing inhibition of root elongation and nutrient uptake and loss of cell viability in growing roots (Inoue *et al.,* 2000; Koyama *et al.*, 2001; Lazof and Holland, 1999; Smith and Krikorion, 1992). Both Al and H, in high concentrations, are considered intrinsic toxicants, causing direct and specific toxic effects in plants (Kidd and Proctor, 2001; Kinraide, 2003). Aluminium has been shown to inhibit the proton pumps in the root elongation zone, interfering in this way with apoplast and cytosol pH, nutrient uptake and root growth (Ahn *et al.*, 2002; Lindberg and Strid, 1997).

Proton in- and efflux over the plasma membrane depend largely on the cell's nitrogen nutrition: for reasons of charge balance, the cell takes up protons together with NO_3^- and it must pump out protons, when assimilating NH_4^+ (Schubert and Yan, 1996). Preference for either of the two nitrogen forms is species specific but generally plants prefer the energetically more efficient NH_4 (Taylor and Bloom, 1998; Von Wiren *et al.,* 1997). Considerable changes in rhizosphere pH have been reported, depending on initial pH and N nutrition (Kosegarten *et al.*, 1999; Schöttelndreier and Falkengren-Grerup, 1999). Nitrogen uptake and proton flux will vary in time and in location along the root axis, though the root elongation zone in maize showed a continued net H^+ efflux, regardless of nitrogen form (Taylor and Bloom, 1998). In short, nitrogen nutrition can direct the pH in the root apoplast and in the rhizosphere, which in turn can influence the activity and accumulation of Al in the (apoplast) solution (Keltjens, 1997; Tan *et al.*, 1992b). At the same time,

Al can reduce nitrogen uptake through inhibition of the H^+-ATPases and possibly even nitrate reduction, affecting H^+ fluxes (Galvez and Clark, 1991; Lidon *et al.*, 1998).

A plant defence mechanism against Al toxicity in the form of an induced increase in rhizosphere pH, which would decrease Al^{3+} activity, is not to be expected in those plants that preferentially take up NH_4 (Keltjens, 1997). A more effective way for a plant to reduce Al effects to the root is exudation of chelators, like organic acid anions and phenolic compounds (Barcelo and Poschenrieder, 2002; Keltjens, 1997). However, the effective chelation by an anion will depend on its degree of protonation and therefore depend on solution pH.

Effects of aluminium and low pH on plant growth and nutrient uptake are notoriously difficult to distinguish, since Al toxicity only occurs when the pH of the rhizosphere decreases below 5 (Kinraide, 2003). Plants can be subjected to low pH treatments to study the effect of a high proton concentration but Al toxicity studies will always include both Al^{3+} and H^+. Yet it is important for a correct interpretation of Al toxicity to separate the effects and to understand specific interactions between Al and nutrient cations.

The objective of the experiments, described in this paper, was to evaluate and separate the effects of Al and pH on plant growth and nutrient uptake, particularly the uptake of the micronutrients Cu and Zn. For this purpose, tomato and wheat seedlings were grown on nutrient solution and subjected to different pH regimes. Plant species were chosen as examples of di- and monocotelydonous plants, which generally differ in cell wall binding capacity for Al. In the first part this is done by imposing two different pH levels on the nutrient solution, in the second part by having the plants create a difference in apoplast pH through differences in nitrogen nutrition. Also in the first part, an Al-chelator, ethylenediamine tetraacetic acid (EDTA), is added as treatment to study the effect of Al in complexed form and to evaluate the effectiveness of this Al-complexation at different pH levels. EDTA was used as a stable substitute for compounds like citrate, malate and oxalate, well known as chelators exuded by several plants species grown under conditions of Al stress (Ma, 2000; Ryan *et al.*, 1995; Zheng *et al.*, 1998).

Materials and Methods

Plant material

Seeds of tomato (*Lycopersicon esculentum* L. cv. Moneymaker) and summer wheat (*Triticum aestivum* L. cv. Minaret) were germinated for 10 (tomato) or 7 days (wheat) in moist quartz sand, at 20°C. Subsequently, seedlings were transferred to 50 L containers with a standard nutrient solution, in climate controlled rooms at a regime of 20°C, 16/8 hours light/dark (light intensity was 70 W m^{-2}) and 80% relative air humidity. The standard nutrient solution consisted of (in m*M*): Ca (1), K (2), Mg (0.25), N (4, as NO_3^-), P (0.075) and S (0.25), and trace elements (in μ*M*): Fe (95; as Fe-EDTA), B (46), Zn (0.8), Mn (9), Cu (0.3) and Mo (0.1). Solution pH was kept at pH 5.5 by automatic titration. The pH was lowered to pH 4 after 7 days of pre-culture, one day prior to the start of the experiments; nutrient solutions were refreshed twice a week.

Experimental set-up

The effect of pH and EDTA on Al accumulation and -toxicity (exp. 1)

The first experiment was set up to expose the two plant species simultaneously to 8 different treatments, in a complete factorial design with 3 variables: solution pH (4.25 and 3.75), Al (0 and 100 μM) and EDTA (0 and 100 μM). For this, 30 tomato and 30 wheat seedlings were grown together for 14 days on each of eight 50 L containers with standard nutrient solution, one container per treatment. Solutions were refreshed twice a week and pH was kept at the set levels by pH stat units. After 11 days, approximately half the tomato plants were removed from each container to prevent mutual shading.

Before the start of the experiment, equally sized plant samples were taken from each container to a total of 32 tomato and 16 wheat plants. The samples were split into two equal groups per plant species for analysis. At the final harvest after 14 days sets of plants were taken in duplicate from each container. Sample sizes were identical for the two pH levels but more tomato plants were taken from the treatments with Al and with EDTA without Al, in order to obtain sufficient biomass for chemical analysis.

The effect of nitrogen form on nutrient uptake at low Al exposure (exp. 2)

For the second experiment tomato and wheat seedlings were exposed to 50 μM Al and a fixed pH of 4 in the bulk solution, with pure NO_3 (both plant species), pure NH_4 (wheat) or NH_4NO_3 (tomato) as N-sources to create differences in apoplast pH. Tomato is too sensitive to NH_4 to have it as single N source and would most likely suffer from chlorosis and reduced growth (Britto and Kronzucker, 2002). Wheat has a much higher tolerance to NH_4. Seedlings were transferred after pre-growth to eight 20 L containers, with 48 (tomato) or 51 (wheat) plants per container and subsequently exposed for 7 days to a nutrient solution at pH 4, with or without added Al. Treatments consisted of 2 Al concentrations (0 and 50 μM) and 2 N-sources: NO_3 and NH_4NO_3 for tomato and NO_3 and NH_4 for wheat, in a complete factorial design with two replicates. For the NO_3 treatment the standard nutrient solution from pre-growth was used. The NH_4 containing nutrient solutions were similar to the standard solution, except that the 4 mM NO_3 was replaced by an equal N concentration in the form of NH_4NO_3 or NH_4 and that the solution had a higher concentration of Cl and SO_4. Two samples of 18 wheat and three samples of 19 tomato plants were taken before Al exposure started. For the final harvest two samples were taken per plant species and per treatment. Sample sizes were 15 and 18 for tomato and 24 and 27 plants for wheat.

NO_3 and NH_4 depletion from the nutrient solutions was measured, as well as the acid/alkali titration to maintain the pH at 4, to monitor the influence of the N-nutrition on the root H^+ or OH^- exudation. Solution pH was measured and automatically adjusted to pH 4 every 15 minutes.

Activity of Al^{3+} in the nutrient solutions, with the different pH levels and N-sources, is calculated with ECOSAT (Equilibrium Calculation Of Speciation And Transport (Keizer *et al.* (1992)).

Plant analysis

Growth analysis: plant samples were separated into shoot and root parts and weighed. The roots were rinsed for 60 sec in demineralised water and blotted dry. Portions of 500-700 mg of fresh root material were taken for measuring root length, using the method of Newman (1966). The shoot and root material was oven dried, overnight at 70°C, and subsequently weighed.

Chemical analysis: samples of the dry plant material were analysed for chemical composition after a microwave HF-HNO_3-H_2O_2 digestion (Novozamsky *et al.*, 1996). Total concentrations of Al, Ca, Mg, K, Zn, Cu, Mn, P, Fe and S in the digest were measured with inductively coupled plasma-atomic emission spectrometry (ICP-AES). Total tissue concentrations of Al, Cu, Zn and Mn in experiment 2 were measured with ICP-MS (ICP-mass spectrometry). Al and nutrient concentrations in shoot or root are expressed in μmol $(g\ DM)^{-1}$. Cl uptake in the second experiment was derived from Cl depletion of the nutrient solution. N in the nutrient solution was measured by segmented-flow analysis.

Statistical analysis

Means of the results were compared with univariate Analysis of Variance (ANOVA) and the Tukey multiple comparison procedure (SPSS 8.0). Significantly different (0.05 level) results were grouped into homogeneous subsets. Interaction between factors was tested with two-way univariate ANOVA.

Results

The effect of solution pH and EDTA on Al toxicity

Effects of Al(-EDTA) and pH on growth in tomato and wheat seedlings

Aluminium decreased dry matter production and root elongation severely in both plant species in the first experiment, with a relatively larger effect in tomato than in wheat (fig. 1, treatment 100-0). The pH had no effect on growth in tomato in control or Al treated plants, whereas a lower pH caused a larger decrease in dry matter production in wheat roots. At pH 3.75, shoot dry matter production and root length increased in control plants of wheat, compared to plants at the higher pH; this increase did not occur in Al treated plants.

In tomato, addition of EDTA together with Al prevented the negative effects of Al on dry matter production and even significantly increased its root length at pH 4.25. However, at pH 3.75 this beneficial effect was very limited (treatment 100-100). EDTA did not counter-act the Al effects in wheat at either of the two pH levels. EDTA in the nutrient solution without Al present (treatment 0-100) had negative effects on growth which were similar to those of Al, in both plant species.

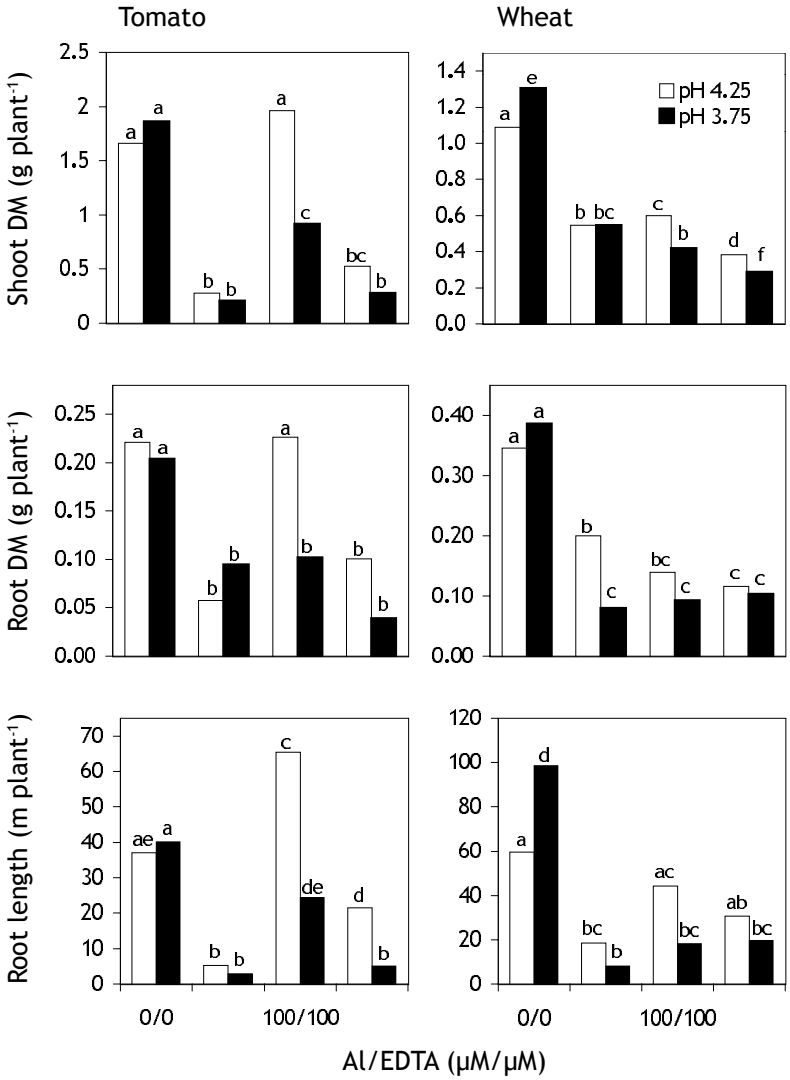

Figure 1: *Shoot and root dry matter and root length in tomato and wheat, after exposure to nutrient solution (control, 0-0) or nutrient solution containing 100 µM $AlCl_3$ (100-0), 100 µM Na_2-EDTA (0-100), or a combination of Al and EDTA (100-100); pH was 4.25 or 3.75. Identical letters in each graph indicate that the results are not significantly different at the 0.05 level.*

Effects of Al(-EDTA) and pH on nutrient uptake

Cation concentrations in shoots and roots in the different treatments were pairwise compared. Al and lower pH significantly decreased the Ca, Mg, K and Mn concentration in tomato shoots, both separately and synergistically (table 1A). Al significantly decreased the tomato root concentration of Mg, K and Mn at both pH levels; Al only significantly decreased Ca in these roots at pH 3.75. Analysis indicated significant interaction between the factors Al and pH for the Al and Mg concentrations in the root.

In wheat shoots and roots, Al significantly decreased most nutrient concentrations at both pH levels, with the clear exception of root Ca (table 1B, treatment 100-0). The pH alone did not effect shoot concentrations of Ca and K, yet pH and Al showed an interaction when combined, decreasing the nutrient concentrations more at pH 3.75 than Al had done at pH 4.25. A change in pH did not change Mg in the shoot, in the presence or absence of Al. Both Al and lower pH decreased Mg concentration in the root but the pH change did not show interaction with the Al effect. Zn and Cu in the wheat shoots were both decreased by Al but not affected by pH. Nevertheless, analysis indicated interaction between Al and pH for both Zn and Cu: Zn was more reduced at lower pH, whereas Cu concentration was less affected by Al.

Al accumulated in the roots of both plant species but addition of 100 μM EDTA prevented this (table 1, treatment 100-0 and 100-100, respectively). Shoot nutrient concentrations in the latter treatment were generally equal to or higher than those in the –Al treatment. This could be seen at both pH levels, with the exception of Cu and Zn at the lower pH in wheat. Without Al in the nutrient solution, EDTA significantly decreased Zn and Cu concentrations in the whole wheat plant at both pHs (treatment 0-100). EDTA only partially prevented the Al effect on Mg concentration in the wheat root.

treatment	1A: Tomato concentration (µmol (g DM)$^{-1}$)				
pH Al-EDTA	shoot				
pH 4.25	Al	Ca	Mg	K	Mn
0-0	0.0^{a}	632^{a}	184^{ae}	1173^{a}	5.3^{a}
100-0	3.2^{b}	372^{bd}	117^{b}	677^{bc}	3.8^{b}
100:100	0.0^{a}	628^{a}	195^{a}	1107^{a}	5.4^{a}
0-100	0.8^{a}	946^{c}	339^{c}	589^{c}	2.0^{c}
pH 3.75					
0-0	0.0^{a}	473^{ad}	129^{b}	865^{b}	4.1^{b}
100-0	2.3^{b}	246^{b}	69^{d}	521^{c}	2.0^{c}
100:100	0.6^{a}	505^{ad}	148^{be}	1135^{a}	4.8^{ab}
0-100	0.3^{a}	516^{ad}	155^{abe}	506^{c}	1.4^{c}
	root				
pH 4.25	Al	Ca	Mg	K	Mn
0-0	1.2^{a}	82^{a}	104^{a}	1379^{a}	17.5^{a}
100-0	240^{b}	69^{abc}	57^{bc}	995^{b}	4.8^{b}
100:100	4.8^{a}	77^{a}	106^{a}	1612^{c}	16.0^{a}
0-100	1.2^{a}	77^{a}	114^{a}	1389^{a}	7.0^{b}
pH 3.75					
0-0	0.9^{a}	76^{a}	70^{b}	1263^{a}	14.8^{ac}
100-0	97^{c}	56^{c}	51^{c}	893^{b}	3.6^{b}
100:100	6.3^{a}	70^{ab}	64^{bc}	1458^{ac}	12.0^{c}
0-100	1.7^{a}	61^{bc}	64^{bc}	838^{b}	3.6^{b}

Table 1: *Al and nutrient concentration in shoot and root of tomato (1A) and wheat (1B), after 14 days of growth on a nutrient solution containing 0 or 100 µM $AlCl_3$, in combination with 0 or 100 µM Na_2-EDTA and at pH 4.25 or 3.75. Homogeneous subsets, indicated by identical letters, are based on univariate ANOVA and the Tukey multiple comparison procedure, with significant differences at the 0.05 level.*

treatment	1B: Wheat						
pH Al-EDTA	concentration (µmol (g DM)$^{-1}$)						
	shoot						
pH 4.25	Al	Ca	Mg	K	Mn	Zn	Cu
0-0	0.7^{a}	104^{a}	54^{ad}	1523^{a}	4.4^{abcd}	0.85^{a}	0.20^{ac}
100-0	1.4^{a}	74^{b}	20^{b}	1343^{ab}	3.2^{ab}	0.42^{b}	0.08^{b}
100:100	1.5^{a}	107^{ae}	82^{cd}	1550^{ac}	8.1^{e}	0.86^{a}	0.23^{a}
0-100	0.6^{a}	123^{c}	94^{c}	1569^{ac}	7.6^{de}	0.36^{b}	0.11^{b}
pH 3.75							
0-0	0.2^{a}	111^{ace}	52^{a}	1530^{a}	4.2^{abc}	0.89^{a}	0.19^{ac}
100-0	1.6^{a}	34^{d}	14^{b}	1128^{b}	1.9^{a}	0.35^{b}	0.13^{bc}
100:100	1.6^{a}	107^{ae}	68^{acd}	1784^{c}	6.6^{cde}	0.65^{c}	0.08^{b}
0-100	0.8^{a}	119^{ce}	83^{c}	1475^{a}	6.2^{bcde}	0.33^{b}	0.11^{b}
	root						
pH 4.25	Al	Ca	Mg	K	Mn	Zn	Cu
0-0	1.4^{a}	29^{a}	54^{a}	1522^{a}	19.7^{a}	0.54^{a}	0.98^{a}
100-0	320^{b}	30^{a}	22^{bd}	990^{b}	5.2^{b}	0.41^{bc}	0.36^{b}
100:100	9.5^{a}	30^{a}	39^{c}	1440^{a}	43.4^{c}	0.51^{ab}	0.54^{b}
0-100	1.3^{a}	32^{a}	36^{ce}	1613^{a}	7.9^{bd}	0.30^{c}	0.11^{c}
pH 3.75							
0-0	2.0^{a}	24^{a}	44^{c}	1404^{ac}	15.8^{ad}	0.52^{ab}	1.41^{d}
100-0	71^{c}	22^{a}	19^{b}	996^{b}	7.3^{bd}	0.46^{ab}	1.80^{e}
100:100	13^{a}	25^{a}	30de	1451^{a}	16.0^{ad}	0.52^{ab}	0.10^{c}
0-100	1.3^{a}	22^{a}	31^{e}	1158^{bc}	7.0^{bd}	0.33^{c}	0.09^{c}

Activities of Al^{3+} in the nutrient solution with 100 µM $AlCl_3$ -/+ Na_2-EDTA were calculated with ECOSAT. Figure 2 shows the change of the Al^{3+} activity in the solution, which increased in the absence of Na_2-EDTA by 8 µM with a change from pH 4.25 to 3.75. Total activity of monomeric Al species (Al^{3+} and Al-hydroxides, -phosphates and -sulphates) decreased from 74 to 66 µM in that pH range. In the solution with equal concentrations of Al and EDTA, 98% of the Al present was bound and Al^{3+} activity was decreased to < 0.5 µM, at both pH levels.

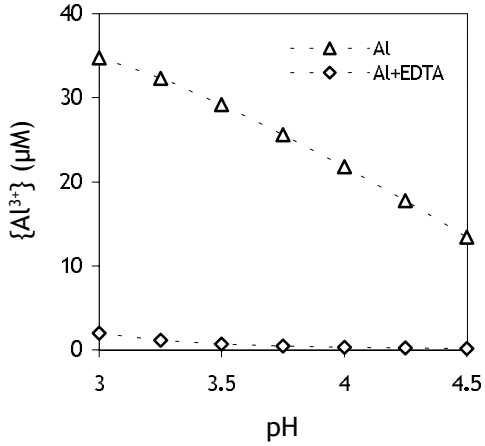

Fig 2: *Calculated equilibrium activity of Al^{3+} in a nutrient solution with 100 µM $AlCl_3$ -/+ 100 µM Na_2-EDTA and NO_3 as N-source, at different pH levels.*

The effect of N form on growth and nutrient uptake at low Al exposure

The main effect of 50 µM $AlCl_3$ on seedling growth was the decrease in root length, which did not depend on N source (fig. 3). Pure NH_4 had a negative effect on wheat root length, though the effect was much smaller than that of Al. Analysis of the nutrient concentrations in shoots and roots with univariate ANOVA showed significant differences between treatments (table 2). When a combination of Al and either NH_4NO_3 or NH_4 was present, significantly less Al accumulated in the roots of both plant species than in the Al treatment with NO_3. In both experiments 1 and 2, P and Fe accumulated in the root when Al was present at a relatively higher pH. This effect disappeared as soon as Al accumulation was reduced by lower pH or NH_4 treatment (data not shown).

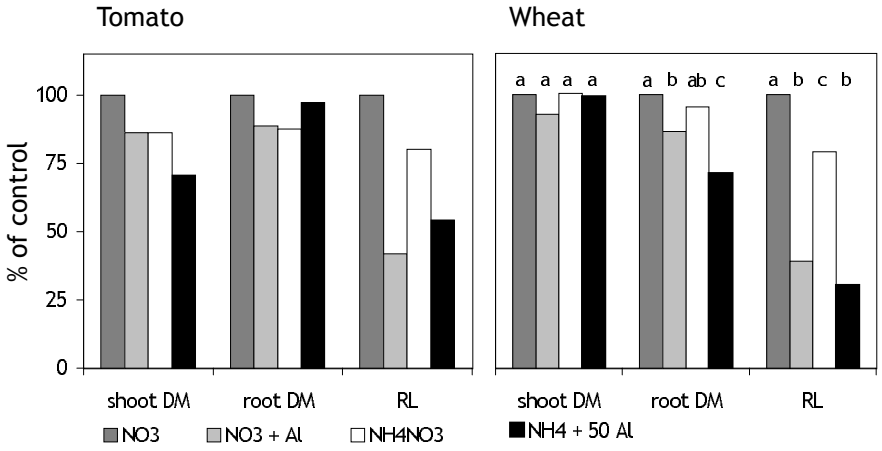

Figure 3: *Shoot and root dry matter and root length in tomato and wheat after 7 days exposure to a nutrient containing 0 or 50µM $AlCl_3$ and different nitrogen sources. N sources were NO_3 or NH_4NO_3 for tomato and NO_3 or NH_4 for wheat. Results are given as % of the result for the first treatment, 0 Al and with NO_3 as N source. Identical letters in the graph indicate that the results for that growth parameter are not significantly different at the 0.05 level. Although mean values varied for tomato, differences were not statistically different at the 0.05 level.*

Al and NH_4NO_3 in the solution decreased shoot and root concentrations of Ca, Mg, K and N in tomato, both separately and synergistically, compared to the –Al/NO_3 treatment. Al and N-source were interacting factors in the analysis for all these concentrations, except for Mg in the root. In contrast to this were the Zn and Cu concentrations in the tomato shoot: no effect of N-source or interaction between Al and N-source.

treatment	**Tomato** concentration (µmol (g DM)$^{-1}$)						
shoot	Al	Ca	Mg	K	Mn	Zn	Cu
NO_3	0.6^{a}	403^{a}	194^{a}	1805^{a}	4.9^{a}	0.69^{a}	0.24^{a}
+50 µM Al	1.6^{b}	313^{b}	106^{b}	708^{bc}	3.2^{b}	0.39^{b}	0.09^{b}
NH_4NO_3	0.6^{a}	238^{c}	136^{c}	1032^{b}	3.1^{bc}	0.57^{ab}	0.21^{a}
+ 50 µM Al	2.0^{c}	206^{d}	87^{b}	437^{c}	2.6^{c}	0.43^{b}	0.10^{b}
root							
NO_3	9.2^{a}	77^{a}	88^{a}	1570^{a}	12.3^{a}	2.04^{a}	0.70^{a}
+ 50 µM Al	71^{b}	59^{b}	56^{b}	855^{b}	4.6^{b}	0.98^{b}	0.25^{a}
NH_4NO_3	6.7^{a}	59^{b}	67^{c}	1020^{b}	8.1^{c}	2.26^{a}	0.62^{a}
+ 50 µM Al	39^{c}	55^{b}	41^{d}	613^{c}	3.7^{b}	1.19^{b}	0.32^{a}

treatment	**Wheat** concentration (µmol (g DM)$^{-1}$)						
shoot	Al	Ca	Mg	K	Mn	Zn	Cu
NO_3	1.4^{a}	116^{a}	47^{a}	1458^{a}	2.8^{a}	0.78^{a}	0.16^{a}
+ 50 µM Al	1.1^{a}	90^{b}	27^{b}	1393^{ab}	2.2^{b}	0.41^{b}	0.09^{b}
NH_4	0.8^{a}	78^{c}	42^{c}	1337^{bc}	2.1^{b}	0.71^{c}	0.14^{a}
+ 50 µM Al	1.0^{a}	69^{d}	27^{b}	1081^{c}	2.1^{b}	0.55^{d}	0.14^{a}
root							
NO_3	3.6^{a}	27^{a}	41^{a}	1134^{a}	5.7^{a}	0.64^{a}	0.31^{a}
+ 50 µM Al	32^{b}	24^{a}	27^{b}	981^{b}	4.2^{b}	0.54^{a}	0.20^{b}
NH_4	3.9^{a}	16^{b}	26^{b}	924^{b}	4.1^{b}	0.58^{a}	0.21^{ab}
+ 50 µM Al	13^{c}	16^{b}	16^{c}	688^{c}	3.0^{c}	0.53^{a}	0.22^{ab}

Table 2: *Al and nutrient concentration in shoot and root of tomato and wheat, after 7 days of growth on a nutrient solution containing 0 or 50 µM $AlCl_3$, at pH 4. N-source in the nutrient solution was NO_3 or NH_4NO_3 for tomato and NO_3 or NH_4 for wheat. Homogeneous subsets, indicated by identical letters, are based on univariate ANOVA and the Tukey multiple comparison procedure, with significant differences at the 0.05 level.*

In wheat plants, both Al and N-source (NH_4) in the solution had a significantly negative effect on most nutrient concentrations. Al and N-source effect interacted in establishing all shoot concentrations, whereas in the root the two factors only interacted in causing a decrease in concentration of K and Cu.

A difference in N-source had little to no effect on Cu and Zn concentrations in the shoot, yet analysis indicated an interaction between the two factors. Interaction between Al and N-source in the case of shoot Cu and Zn concentration consisted of partial or full prevention of the Al effect in the presence of NH_4.

ECOSAT equilibrium calculation showed that Al^{3+} activity in the nutrient solution changed with N source but that the differences were not very large and decreased with increasing pH (fig. 4). Total activity of monomeric Al-species was between 35 and 39 µM in all solutions (data not shown). The NH_4 solution contained relatively less Al^{3+} and Al-phosphates and more Al-sulphates, as a result of differences in solution anion composition.

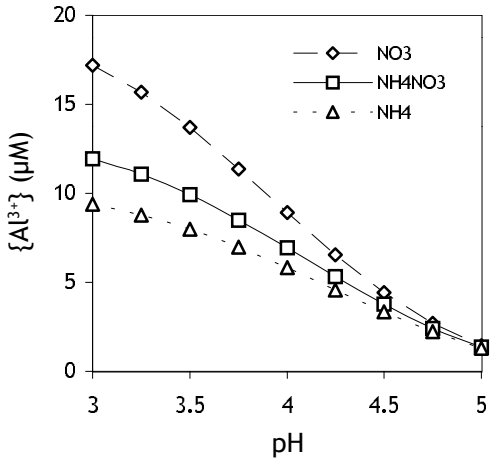

Fig 4: *Calculated equilibrium activity of Al^{3+} in nutrient solutions with 50 µM $AlCl_3$ and different N sources, as used in experiment 2. Al^{3+} activity shifted with change in pH and nutrient solution composition.*

Total nitrogen consumption per g dry matter increase was generally the same with both N-sources (table 3). Though many plants preferentially take up NH_4, tomato in this experiment took up 1.5-2 times as much NO_3 as NH_4 (table 3). Because of this, the tomato plants with NH_4NO_3 as N source did not acidify the nutrient solution, as was intended. Still, with tomato, a range of pH levels was created from pH 4 upward with differences in N uptake. The solution pH changes were smaller with a combined uptake of NO_3 and NH_4, since the H^+ efflux from NH_4 uptake compensated for part of the NO_3 uptake. At the same time, Al reduced total N uptake by > 50% when only NO_3 was present, to 40% from the combined N forms. Effectively, the pH changed in the order NO_3 > NO_3 + Al > NO_3/NH_4 > NO_3/NH_4 + Al.

treatment	Tomato uptake (meq g^{-1}) cation	anion*	NO_3	NH_4	sum	titration (meq H^+ g^{-1})
NO_3	2.9	0.5	4.7	-	2.3-	2.8
+ 50 µM Al	1.3	0.3	2.3	-	1.3-	2.1
NH_4NO_3	1.5	0.5	3.3	1.8	0.5-	1.0
+ 50 µM Al	0.6	0.3	1.8	1.2	0.3-	0.6

treatment	Wheat uptake (meq g^{-1}) cation	anion	NO_3	NH_4	sum	titration (meq g^{-1}) H^+	OH^-
NO_3	1.7	0.5	4.5	-	3.3-	3.8	-
+ 50 µM Al	1.5	0.4	4.1	-	3.0-	3.2	-
NH_4	1.4	1.5	-	4.5	4.4	-	4.9
+ 50 µM Al	1.1	1.3	-	4.1	3.9	-	4.2

* Cl and SO_4^- not measured

Table 3: *Uptake of total cation (Ca^{2+}, Mg^{2+}, K^+), anion (H_2PO4^-, $SO4^-$, Cl^-), NO_3^- and NH_4^+ in meq per g increase in tomato and wheat total dry matter. Nutrient solutions contained NO_3, NH_4 or a combination of both as N source and 0 or 50 µM $AlCl_3$. The sum gives the approximate net charge increase per g plant, which needs to be balanced out by proton uptake or extrusion. Solution pH was measured and adjusted every 15 min. Figures in the last column refer to the measured total amounts of titrated acid or alkali per g dry matter increase, for maintaining the pH at 4.*

Al decreased total N uptake to the same extent in both NO_3 and in NH_4 grown wheat plants (table 3). Like in tomato, both Al and NH_4 decreased cation uptake. NO_3 grown plants took up an excess of negative charge, which was compensated by a net H^+ uptake, whereas the NH_4 grown plants needed to compensate by proton extrusion. This will have resulted in an apoplast pH increase and decrease with NO_3 and NH_4 nutrition, respectively. The maximum changes in pH, which were measured in the nutrient solutions, were +0.15 (–Al) and +0.07 (+Al) for the NO_3 treatments, while the changes with NH_4 in the solution were -0.19 (–Al) and -0.13 (+Al).

Discussion

The results of the experiments described here form no exception when it comes to difficulty in separating Al and H effects on plants. Yet, the factorial design and the use of nutrient solution instead of soil as rooting medium have enabled us to evaluate the relative importance of both toxicants in their effect on plant growth and nutrient uptake.

Both an imposed lower pH in the bulk solution and an induced (relative) decrease in rhizosphere and apoplast pH as a result of NH_4 nutrition decreased the Al accumulation in the root. Yet the plants did not benefit from this change, neither in growth, nor in nutrient uptake. On the contrary, root dry matter production in wheat decreased even more with Al at the lower pH or with NH_4 as N source. At low pH, protons are a strong competitor for binding sites in the root apoplast and less Al will adsorb and accumulate. Al accumulation reflects the high affinity of apoplast binding sites for Al^{3+} and also the ratio between activity of Al^{3+} and H^+ (or another dominant apoplast cation, like Ca^{2+}) in the root apoplast. Binding of Al to root cell walls is considered an important factor in the Al-induced inhibition of root elongation.

The actual pH in the root apoplast will influence the effect of Al on nutrient uptake. The nutrient solution and apoplast pH in all experiments was subject to temporary changes as a result of nitrogen uptake. The plants in exp. 1 increased the pH as a consequence of NO_3 uptake. Nitrate consumption was not measured in this experiment but exp. 2 gives an indication for the extent of the root proton uptake or extrusion with increase in biomass. The only considerable solution pH change in the presence of Al in exp. 1 was estimated for wheat, which would have elevated the pH from 4.25 to 4.35. The actual change in pH may have been smaller, since Al reduces uptake of N and the Al concentration in exp. 1 was higher. The wheat control plants (–Al) also caused the largest estimated pH increase in solution (to pH 4.55), as expected with a higher NO_3 uptake and with a relatively larger effect at a higher pH.

All pH measurements and calculations were done in the bulk nutrient solution and the pH changes in the bulk solution are expected to reflect the major changes in the root apoplast pH. Nevertheless, local and transient pH changes at the root surface and in the root apoplast must have been substantially larger, depending on plant species and treatment, to create a pH change in the bulk solution. The uptake of e.g. NH_4 by wheat and the subsequent proton efflux are expected to have strongly acidified the root apoplast. Since the bulk solution pH already was as low as 3.81 (–Al) and 3.87 (+Al), the apoplast pH in this treatment was probably substantially below the pH 3.75, which was used in exp. 1 and which subsequently increased as a result of NO_3 uptake.

The most straightforward specific Al effects on nutrient uptake can be recognised in those nutrient concentrations that are not affected by a change in solution pH alone but which are decreased by Al. The most consistent effect of Al on nutrient uptake and translocation was the decrease in Cu and Zn concentrations in both plant species. A change in N-source or pH did not have an effect on the Cu and Zn concentrations in wheat shoots, yet an interaction was found to exist between Al on the one hand and pH or NH_4 in the solution on the other hand. The effect of Al on Cu shoot concentration was partially prevented in exp. 1 and absent in exp. 2. A high proton concentration appears to be able to prevent the Al effect on Cu uptake, assuming that the apoplast pH was lower with the uptake of NH_4.

Al^{3+} activity in the second experiment was much lower, while at the same time the proton activity was most likely substantially higher than in exp. 1. The decrease in $\{Al^{3+}\}\{H^{+}\}^{-1}$ ratio will have decreased the interaction of Al^{3+} with apoplast binding sites. If an uptake mechanism for Cu is inhibited by Al but insensitive to pH change, a decrease in Al competition, as a result of lower $\{Al^{3+}\}\{H^{+}\}^{-1}$ ratio, may improve Cu uptake. Additionally, the cell wall itself may have a role in Cu uptake. More than half of the Cu in the root may be bound to cell wall sites (Kochian, 1991) and Al severely decreases cell wall binding of Cu (chapter 3 of this thesis). Prevention of Al binding to the cell wall improved Cu adsorption to these sites in isolated cell walls. If a functional connection between cell wall binding and uptake exists, then a low pH and low Al accumulation could be beneficial for Cu uptake. The data in these experiments are not conclusive with respect to Cu uptake in the root: low pH strongly increased Cu concentration in the wheat roots in exp. 1. Yet the presumably very low pH in exp. 2 did not improve Cu root concentration compared to the Al treatment; this aspect needs further study.

The presence of NH_4 in the solution did not change the Al effect on the tomato shoot concentrations of Cu and Zn. The limited acidification of the tomato root apoplast in exp. 2 may be the explanation for the absence of a positive effect like in wheat. Zn concentrations were, like Cu, not sensitive to the change in pH or N-source. Whereas the low pH in exp. 1 did not improve Zn uptake, the Al effect was at least partially prevented when NH_4 was the N-source. A lower affinity for Zn compared to Cu, like it is found for cell wall binding sites, may be the reason that Zn uptake could profit less from the low pH than Cu did. Unfortunately no data were available for Cu and Zn uptake in tomato under Al exposure at the two pH levels.

Al decreased Mg concentration in all tissues of both plant species, a known symptom in Al phytotoxicity. Although the Al^{3+}activity in the nutrient solution increased from 18 μM at pH 4.25 to 26 μM at 3.75, the decrease in Mg concentration in wheat shoots was not significantly different at the two pH levels. The Al effect on Mg was similar in experiment 2, where both Al root accumulation and Al^{3+} activity in the solution were much lower. However, analysis indicated interaction in exp. 2 between Al and the type of N-source, since both factors decreased Mg in wheat shoot separately but the effects were not additive.

A similar effect is visible in Mn concentrations in the same wheat shoots and in Mg in tomato roots in exp. 1. Interaction in these cases may mean something different from the effect in Cu and Zn uptake. If both H^+ and Al^{3+} interfere with the same factor in an uptake mechanism, the effects may not be additive and if the effect of Al dominates over the H effect, a very large decrease in pH is needed to give a visible change. The decrease in Mg concentration in wheat shoots appears to be mainly, if not solely, caused by Al. For tomato, the results are less straightforward. Although the larger decrease in shoot Mg at pH 3.75 can be explained by the increase in Al^{3+}activity in the solution, the Mg concentration also proved to be very sensitive to apoplast acidification and a direct pH effect cannot be excluded.

Ca and K concentrations in the wheat plants were insensitive to the pH change in exp. 1, in the absence of Al. However, Al decreased shoot Ca and K concentrations at pH 3.75 significantly more than it had done at pH 4.25 and analysis shows interaction between Al and pH effect. In this case interaction caused aggravation of the Al effect and in exp. 1 it may be directly linked to the increase in Al^{3+}activity with decreasing pH. Another possibility may be that e.g. the presence of Al sensitises the uptake system for low pH. The strong acidification in response to NH_4 uptake in the –Al plants of exp. 2 did have a negative effect on wheat Ca and K concentrations. The high proton activity acted synergistically and interactively with Al in reducing Ca and K uptake and translocation.

All concentrations of nutrient cations in tomato shoots, except for Zn and Cu, proved to be sensitive to pH changes in both experiments. Although Ca, K and Mn in the tomato roots were not sensitive to the pH change in exp. 1, the relatively stronger acidification in the apoplast of NH_4NO_3 treated plants in exp. 2 did cause a decrease in nutrient uptake. This NH_4NO_3 effect is also visible in the Al-treated plants. A decrease in pH to 3.75 dramatically decreased the beneficial effect of EDTA on tomato growth. This is not likely to be due to a decreased ability of EDTA to chelate Al^{3+}, thus allowing Al^{3+} to affect growth, since calculations with ECOSAT indicate no change in Al speciation in this pH range. EDTA could not improve growth of wheat plants at either pH level, though in all cases Al^{3+} activity was decreased by > 99%. The negative effect appears to be EDTA related and is most likely due to inhibition of Cu and/or Zn uptake.

EDTA strongly decreases Cu and Zn activity in the nutrient solution, both at pH 4.25 and 3.75 (ECOSAT). Though Cu and Zn concentrations were not deficient in wheat shoots, uptake was in many cases limited, especially when bearing in mind that the plants in question were much smaller than the controls. Moreover, wheat is known to be very sensitive to Cu deficiency, which could explain the decrease in growth. A similar effect may have limited growth in tomato at pH 3.75, although no explanation can be given for the difference at the two pH levels. The use of an Al-chelator, like Na_2-EDTA, in this Al toxicity study was effective in reducing most Al-induced effects on nutrient uptake. Yet the interference of the chelator with trace-nutrient uptake caused undesirable side effects in plant growth.

The effect of Al on nutrient uptake is expected to be specific, when direct interaction with ion channels is involved, and more general when it is through cation competition for apoplast binding sites or through a change in membrane potential. The effect of Al on Cu and Zn shoot concentration was independent of pH, within the pH range 3.75 - 4.25 used in the experiment. This pH independence, the absence of response to the pH related change in Al^{3+} activity and the fact that uptake did not respond to the type of N -source indicate that the effect on Cu and Zn concentration is specific for Al. The Al-induced decrease in Mg concentration in the wheat shoots was likewise independent of ambient pH and thought to be an Al-specific response. The response of Ca and K in the wheat shoot, of which the concentration decreased with increasing Al^{3+} activity but did not respond to pH decrease itself, may also be Al specific. To fully understand the effect of Al on nutrient uptake, the system will have to be studied at the cellular or at the transporter level. In summary, Al induced a specific decrease in Cu and Zn uptake and translocation, independent of pH, in both plant species. In wheat shoots, the response of Mg concentration was considered Al specific, while Ca and K concentrations responded to Al and not to pH in the pH range 3.75 - 4.25 but were sensitive to further pH decreases with the NH_4 treatment. Nutrient uptake in tomato was generally sensitive to both low pH and Al. Al and pH or N-source were factors in the nutrient uptake that could interact in several different ways, leading to alleviation ofAl toxicity or to (non-) additive toxic effects on nutrient uptake.

Although a low pH and increased proton-efflux as a result of NH_4-nutrition both accomplished a significant decrease in root Al accumulation, this apparently only benefited the trace elements Cu and Zn.

6

Citrate alleviates aluminium toxicity effects on plant growth but only partially prevents effects on nutrient uptake in wheat (*Triticum aestivum* L.)

Abstract

Organic acids in the soil and in plant root exudates can strongly reduce the toxic effects of aluminium on plant growth. Low molecular weight organic acids, like citrate and malate, can chelate free Al^{3+} and decrease its interaction with the plant root. When present in equal concentrations to Al, citrate can usually prevent the typical Al-induced inhibition of root elongation. It is less known if this alleviation extends to another toxic effect of Al: inhibition of nutrient uptake.

A nutrient film technique with continuously refreshed solutions was used to study the effect of citrate on Al toxicity. This technique was chosen to prevent a high microbial activity and subsequent organic anion degradation. Wheat seedlings were exposed for 110 hrs to nutrient solution with 100 μM $AlCl_3$, 100 μM citrate or a combination of both. Citrate allowed Al to accumulate to a relatively high concentration in the wheat roots, probably as an Al-citrate complex. The citrate in the solution did prevent the Al-induced decrease in root dry matter production and root elongation. It did not, however, accomplish full nutrient uptake and net root uptake of Mg, Cu, Zn and Fe were still severely reduced. Whereas uptake of Mg, Cu and Zn were most likely affected by the accumulated Al in the root, the uptake of Cu, Zn and additionally Fe are expected to have been influenced by the high citrate concentration. The latter would mean a possible negative side effect of citrate exudation if active as an Al tolerance mechanism.

Jacqueline W.M. Postma and Willem G. Keltjens
To be submitted

Introduction

Plants growing on acid soils are often exposed to phytotoxic concentrations of aluminium (Al) in the soil solution, which limit growth and nutrient uptake. The presence of organic matter and low molecular weight organic anions in the soil is known to benefit plants by binding Al^{3+} and thus decreasing its activity and toxic effects (Harper *et al.*, 1995; Ritchie, 1995). A plant can synthesise and release organic anions itself in response to Al in the (soil) solution, depending on plant species or cultivar and on Al-tolerance (Gaume *et al.*, 2001; Larsen *et al.*, 1998; Pellet *et al.*, 1995). Many plants adapted to an acid environment have the capacity to exude small organic anions, like citrate, oxalate and malate, as a mechanism to tolerate toxic Al concentrations (Bruun *et al.*, 2001; Schöttelndreier *et al.*, 2001). Exudation can occur immediately after exposure to Al, like release of malate from wheat roots, through induction of an anion channel in the plasma membrane (Kollmeier *et al.*, 2001; Li *et al.*, 2000). Maize plants responded to Al by both immediate exudation of citrate and a slower responding increase in organic acid synthesis (Li *et al.*, 2000; Piñeros *et al.*, 2002). There are many examples of the importance of organic anion exudation in Al toxicity. Overproduction of citrate conferred Al tolerance in transgenic tobacco (de la Fuente *et al.*, 1997). Another example is the release of citrate and malate by an Al-tolerant maize, which caused an extrusion of the Al that had entered the root apoplast in the hours before onset of exudation and which at the same time tempered the Al inhibition of root elongation (Jorge and Arruda, 1997). Especially citrate forms a strong complex with Al, decreasing its interaction with plant root surfaces (Blamey *et al.*, 1997; Ginting *et al.*, 1998; Hue *et al.*, 1986; Ostatek-Boczynski, 1995). When present in a ratio of 1:1 citrate:Al, citrate can prevent Al from inhibiting root elongation, the most Al-sensitive growth parameter known in plants (Barceló *et al.*, 1996).

In the past ten years, the study of the role of Al-organic anion complexes in Al-tolerance has focused on the induction of organic acid synthesis and exudation in the plant roots and its effect on the Al-induced growth inhibition. Relatively little attention has been given to the role oforganic acids in counteracting the other aspect of Al toxicity: the inhibition of nutrient uptake.

The question addressed in this chapter is whether complexation by organic anions is sufficient to prevent not only Al-accumulation and growth inhibition in the plant root but also the toxic effects of Al on nutrient uptake and translocation.

Al accumulates in the root apoplast and interferes with nutrient uptake through competition with nutrient cations and through inhibition of ion channels in the plasma membrane (Archambault *et al.*, 1996; Kinraide *et al.*, 1992). A general symptom of Al toxicity is that, after prolonged exposure, a plant becomes Ca and Mg deficient (Jentschke *et al.*, 1991; Kidd and Proctor, 2000). The effect of Al on uptake and translocation of the trace elements Cu and Zn was shown in previous chapters of this thesis. Addition of citrate to a nutrient solution with Al improved the uptake of Mg and prevented an increase in cytosolic Ca concentration, which was induced by Al (Keltjens, 1995; Ma *et al.*, 2002). Transport of Al(-citrate) over the plasma membrane into the cell is very limited and slow, containing the Al in the root apoplast in most plants (Kochian, 1995; Taylor *et al.*, 2000). Exceptions are Al accumulator plants like *Hydrangea macrophylla* and *Melastoma malabathricum* L., which can take up and accumulate Al to mM concentration in the leaves, in the form of organic complexes (Ma *et al.*, 1997a; Watanabe and Osaki, 2001). Organic acids in the plant are also directly involved in nutrient uptake and translocation, next to their role in Al tolerance. Citrate is released by P-deficient plants for mobilisation of bound P in the soil (Hoffland *et al.*, 1989; Geelhoed *et al.*, 1998; Ström *et al.*, 2002). It is also considered to mobilise Ca and Mg from minerals by weathering (Landeweert *et al.*, 2001). Citrate-complexes are common forms for Zn, Mn and Fe in xylem transport, facilitating transport by decreasing cation interaction with apoplast binding sites (Mengel, 1994; Welch, 1995).

In order to study the effect of Al-organic anion complexes on plant growth and nutrient uptake, wheat seedlings were exposed to nutrient solutions at pH 4, containing aluminium, Al-citrate or citric acid. Results of nutrient uptake, dry matter production and root elongation were compared with those of control plants grown on nutrient solution. The use of regular hydroponics in combination with organic acids can lead to extensive microbial activity in the solution and subsequent breakdown of the organic acids.

Though complexation with Al will delay mineralisation, it will still occur (Brynhildsen and Rosswall, 1997). The use of antibiotics on a larger scale or over a longer period of time was not considered feasible, especially since necessary concentrations of antibiotics are often phytotoxic (Kerven *et al.*, 1991). Therefore, in this study an adaptation of the nutrient film technique used by Moorby and Nye (1983) was used, in which a nutrient solution flowing past the roots was continuously refreshed. The rapid breakdown of low molecular weight organic acids in solution was the main reason to use the more stable Na_2-EDTA as a substitute for organic anions in the previous chapters. The intention of the experiment described here was to create a more natural set-up for studying Al-citrate interaction with the plant.

Al will influence plant nutrient concentrations by interfering with nutrient uptake and by affecting dry matter production. The nutrient concentration at the end of an Al exposure experiment will be a resultant of nutrient uptake, the nutrient status of the plant prior to the experiment and nutrient reallocation within the plant. To evaluate the effect of Al on nutrient uptake, regardless of prior nutrient status, the use of a so-called FiNC (Fictitious Nutrient Concentration) is proposed in this study. This approach will be discussed in more detail in Results and Discussion.

Materials and methods

Seeds of wheat (*Triticum aestivum* L. cv. Minaret) were germinated for 2.5 days in aerated demineralised water. Seedling were selected, which had formed approximately 5 mm central root and 2-3 mm lateral roots, and inserted separately at the top ends of 5 mm wide plastic straws, to direct roots into parallel growth. The seedlings were left to grow for 4 days, floating on 1 mM $CaSO_4$, in a climate controlled room at 20 °C, 70% RH and at a 16/8 hrs light/dark rhythm (70 W m^{-2}). An adaptation of the set-up for nutrient film technique by Moorby and Nye (1983) was used for the Al (-citrate) exposure experiment described here. Seedlings were placed in sets of three plants in triangularly shaped channels, formed by partitions in a double-layered polycarbonate sheet, and placed at a 15° slope (fig. 1).

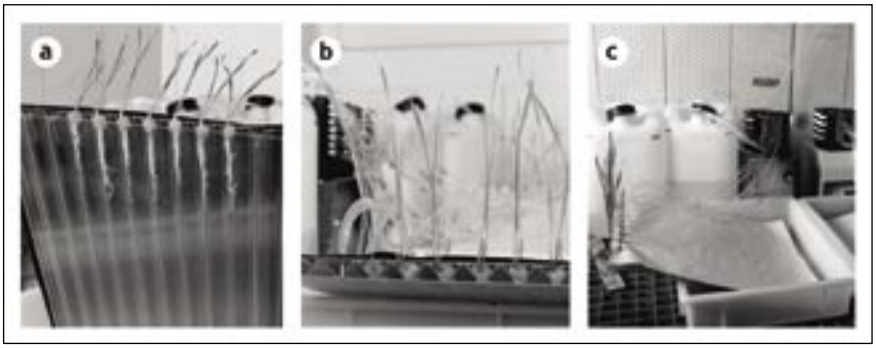

Figure 1: *Set-up for the nutrient film technique for exposure of wheat seedlings to $AlCl_3$, with or without citrate, in a nutrient solution. Wheat seedlings were inserted into triangular channels of a double layered polycarbonate sheet; each channel contained one set of 3 seedlings (a). The sheet was positioned at a 15° slope; inlet for treatment solutions was from the top surface at the onset of the root (b). The surface of the sheet was covered to protect the roots from direct light. Fresh solution was constantly pumped in at the top of each channel with the use of peristaltic pumps (Gilson Miniplus III) (c).*

The sheets were covered to protect the roots from direct light. Plant roots were irrigated continuously, per set of plants, with a complete nutrient solution at pH 5.4 and with the use of peristaltic pumps (Gilson Miniplus III) at a speed of 2.5 mL solution min^{-1}. The nutrient solution consisted of (in mM): Ca (1), K (2), Mg (0.25), N (4, as NO_3^-), P (0.075) and S (0.25), and trace elements (in μM): Fe (95, as Fe-EDTA), B (46), Zn (0.8), Mn (9), Cu (0.3) and Mo (0.1).

After 32 hrs of additional pre-growth seedlings were subjected for 110 hrs to one of the following treatments: nutrient solution (control); nutrient solution + 100 μM citric acid (cit); nutrient solution + 100 μM $AlCl_3$ (Al) or nutrient solution + 100 μM citric acid + 100 μM $AlCl_3$ (Al + cit). During this 110 hrs experimental period, solution pH in all treatments was 4. To maintain the citrate level in the rhizosphere constant at the pre-set level during the whole experimental period, plants were constantly supplied with fresh solution. Each treatment consisted of 4 sets of 3 plants and was performed in duplicate.

Plant material was sampled from pre-grown seedlings at the start of the experiment (day 0) and after 110 hrs, when all plants in the experiment were taken for the final harvest. Plant samples were separated into shoot and root parts and weighed. The roots were rinsed for 60 sec in demineralised water and blotted dry. Portions of 500-700 mg root material were taken for measuring root length, using the method of Newman (1966). The shoot and root material was oven dried, overnight at 70°C, and subsequently weighed. The increase in shoot and root dry matter and the root elongation, since the start of the experiment, were calculated. The specific root length (SRL) was calculated for the samples at the final harvest. Samples of the dry plant material were used for chemical analysis after a microwave HF-HNO_3-H_2O_2 digestion (Novozamsky *et al.*, 1996). Total concentrations of Al, Ca, Mg, K, Zn, Cu, Mn, P and Fe in the digest were measured with inductively coupled plasma-atomic emission spectrometry (ICP-AES) and - mass spectrometry (ICP-MS).

All results for growth parameters and nutrient concentrations are given as the mean value of the 2 duplicates, which each consist of 4 pooled sets of 3 plants. Error bars indicate the standard deviation of the mean. Activity of Al^{3+} and nutrient in the solutions was calculated using ECOSAT (Equilibrium Calculation of Speciation and Transport; Keizer *et al.*, 1992).

Results and Discussion

Aluminium exposed roots showed visible growth inhibition of lateral roots, when compared to the control plants, with the swollen, stubby appearance typical for Al toxicity (fig. 2a and b). Roots from the treatment with Al and citrate were similar in appearance to those of the control plants, with long, slender (lateral) roots; only in one position had some lateral roots stopped growing (fig. 2c). The latter may have been due to a local higher sensitivity to Al because of surface damage. Citrate in the solution, without Al present, did not change the appearance of the roots (not shown).

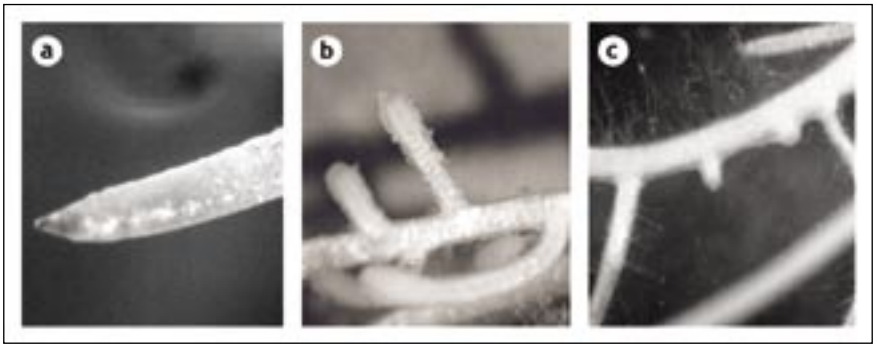

Figure 2: *Root morphology after 110 hrs exposure to nutrient solution containing (a) 0 Al, (b) 100 µM $AlCl_3$ or (c) 100 µM $AlCl_3$ and 100 µM citrate. Al exposed roots had shorter lateral roots with thickened ends, compared to the control plants in (a). Side roots in plants exposed to Al and citrate generally were like in the control plants, though inhibition of lateral root outgrowth occurred in one position (c). (Pictures taken at (a) 50x- and (b and c) 20x-magnification).*

Figure 3 shows the growth parameters for the four treatments. Dry matter production in the shoots was similar in all treatments. Wheat total biomass production responds relatively slowly to Al, as was already shown before in chapter 4 of this thesis. Shoot dry matter contributed the major part in the biomass production, which in chapter 4 did not decrease until 1 week after the start of exposure to 100 µM Al. Root dry matter production in the experiment described here, however, was strongly inhibited by Al. After 110 hrs of exposure to 100 µM Al, the root dry matter increase was only 39% of the control. This effect was to a large extent but not fully prevented by the presence of citrate (Al and Al+cit, respectively). Citrate (cit) itself also caused a decrease, though not as much as the Al did, and the dry matter increase in the citrate and the Al/citrate treatments were very similar. Root elongation, the growth parameter which is usually the most sensitive to Al, was in the presence of Al only 15% of that of the control plants. This effect was also to a large extent prevented in the Al/citrate treated plants, though root elongation did not reach the level of the control plants. Citrate in the solution appeared to have had some specific interaction in the root, since its presence caused a rather large variation in root elongation. Root dry matter increase

and root elongation in the Al/citrate and in the citrate treated plants were equally restrained, resulting in a specific root length (SRL), which was identical to that of the control plants. Al treated plants had a more severe inhibition of root growth than of root biomass production and the SRL was decreased by > 50%. This corresponds with the short and thick roots in fig. 2b.

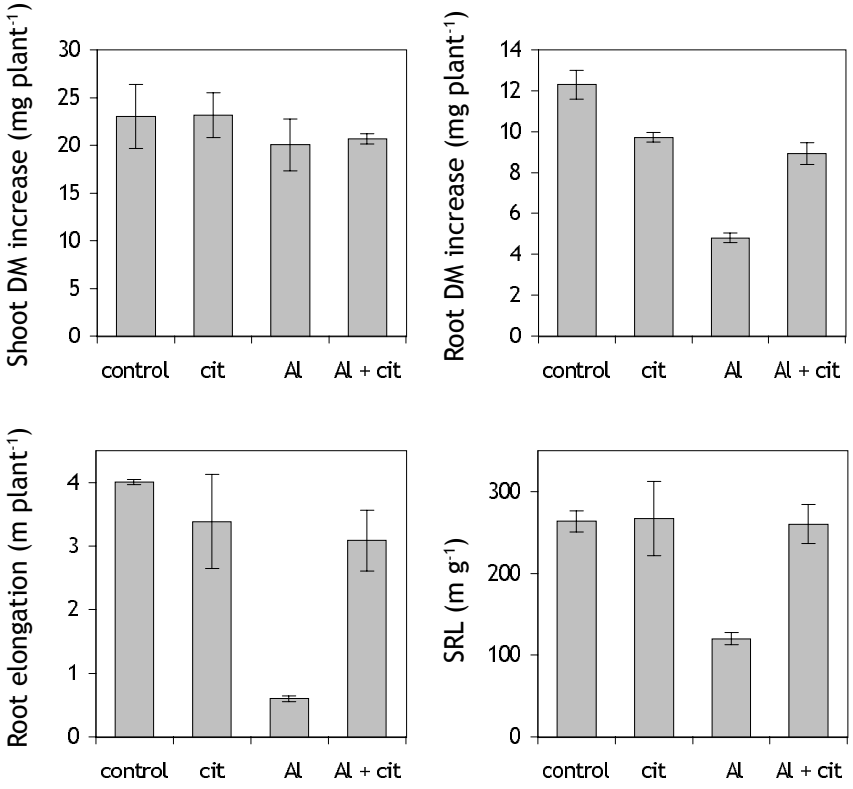

Figure 3: *Increase in shoot and root dry matter and in root length in wheat after 110 hrs growth with solution flow of: nutrient solution or NS (control), NS + 100 µM citrate (cit), NS + 100 µM $AlCl_3$ (Al) or NS + 100 µM $AlCl_3$ + 100 µM citrate (Al+cit). The specific root length is deduced from the root length and weight at the final harvest. Error bars indicate the standard deviation of the mean.*

Aluminium in toxic concentrations generally decreases plant nutrient uptake before it affects dry matter production. To what extent a plant suffers in growth from a decrease in nutrient uptake depends on the one hand on the magnitude of that decrease and on the other hand on the nutritional status of the plant, prior to the decrease in uptake. Nutrient concentrations in a plant can usually vary within a certain range without causing an immediate change in growth. A high nutrient concentration in the plant at the start of an experiment will delay growth inhibition induced by nutrient deficiency, especially in the case of nutrients that are more mobile within a plant. An Al induced decrease in biomass production will also influence the nutrient concentrations in the plant, though this will result in a relative increase in concentration. Final nutrient concentrations in the plant will depend on the balance of inhibition of nutrient uptake and dry matter production.

In order to evaluate the effect of Al on nutrient uptake over the 110 hrs of the experiment, without taking the nutrient status of the plant at the start of the experiment into account, the increase in shoot and root content was calculated for a range of nutrients. Divided by the weight increase per plant part, it gives an idea of what the nutrient concentration in newly formed tissue should be, provided there is no nutrient reallocation and that all nutrients taken up are invested in the new tissue. These assumptions may not be fully correct but a comparison between the Al treated plants and the controls is still valid and the procedure brings out the Al effects more clearly than a final average nutrient concentration would. A so-called Fictitious Nutrient Concentration in newly formed tissue (hereafter called: FiNC) is thus calculated as: (increase in total nutrient per shoot or root) (increase in shoot or root dry matter)$^{-1}$ and is expressed in μmol (g dry matter)$^{-1}$.

Figure 4 shows the results in FiNC per shoot and root for the different nutrients. Al had a large effect on the uptake and translocation of most nutrients: all FiNCs decreased, compared to the control plants, except those of root P and Fe. Root accumulation of these two nutrients occurs often in connection with Al toxicity. The most remarkable Al induced decrease in FiNC is that for root Cu and Zn: the plants showed a net efflux, a decrease in root content, compared to the start samples. Shoot concentrations maintained a reasonable level as a result of root-shoot translocation but uptake into the

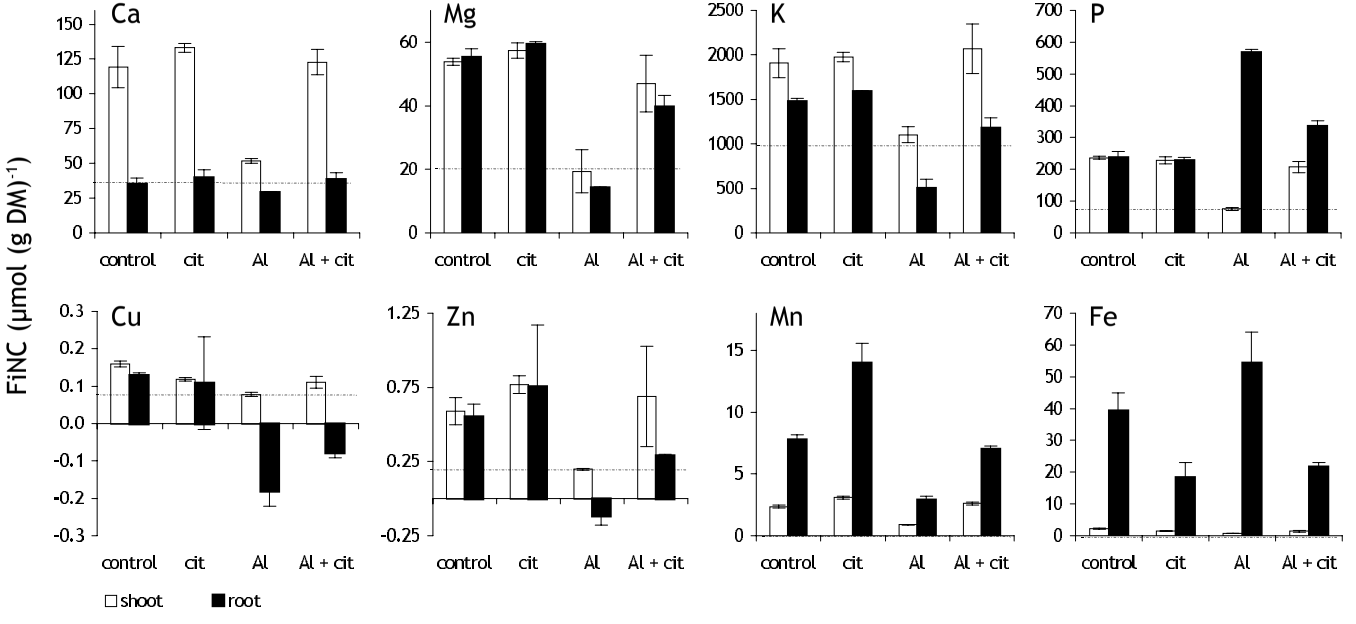

Figure 4: *Nutrient uptake and translocation in wheat seedlings after 110 hrs of treatment with nutrient solution (NS = control), NS + 100 µM citrate (cit), NS + 100 µM $AlCl_3$ (Al) or NS + 100 µM $AlCl_3$ + 100 µM citrate (Al+cit). Nutrient uptake is expressed as a Fictitious Nutrient Concentration (FiNC) in shoot and root: the increase in nutrient content per gram dry matter increase (see text). Dashed lines indicate a deficiency level for shoot nutrient concentration. Error bars indicate the standard deviation of the mean.*

root was inhibited in such a way that less Cu and Zn entered the root than was translocated to the shoot. Citrate reduced the Al effect but a negative net FiNC of Cu in the wheat roots remained. Roots took up Zn in the Al/citrate treatment, though the uptake was still reduced, compared to the control.
Dashed horizontal lines in the graphs in figure 4 indicate critical levels of shoot nutrient concentration: the plant is considered to be deficient at a concentration below the line (de Kreij *et al.*, 1992; Reuter *et al.*, 1997). Deficiency limits are usually set for the above ground parts of a plant and little is known of deficiency levels for root tissues. The shoots of Al exposed plants had become deficient in Mg and Zn and concentrations of Cu and P were just at the critical level. Though strongly decreased by Al, the shoot concentrations for Ca and K were still sufficient. Fe and Mn requirements in wheat shoots are so low that deficiency never occurred. Addition of citrate, together with Al, kept all nutrient concentrations in the shoots above deficiency level and increased most concentrations to values, comparable to the control. However, root concentrations of Mg, K, Zn and especially Cu remained below that in control plants.

Citrate in the solution (cit) had no effect on uptake of the macronutrients (Ca, Mg, K and P). It increased the Mn uptake in the root and decreased that of Fe. The lower Fe concentration, which is also seen in the Al/citrate treatment, could be a consequence of the decrease in Fe^{3+} activity in the solution, due to complexation by citrate. Root concentrations of Cu and Zn were very variable in the Al/citrate treatment and both metals are known to interact with citrate. Zn concentration in the shoots was higher than in the control and Zn is possibly taken up and translocated more easily as a citrate complex. This is not the case for Cu, of which the solution activity is strongly decreased (-95% in the presence of citrate) and uptake is lower, in both citrate and Al/citrate treatment, than in the control plants.

Citrate was chosen for this study for the reason that it is one of the strongest and most common Al chelators found in plants, though wheat generally exudes little citrate. Al-tolerant wheat cultivars can exude malate in response to Al toxicity, which could possibly interfere with the experiment (Delhaize *et al.*, 1993; Pellet *et al.*, 1996). Yet, the cultivar chosen for this work was not specifically tolerant, nor especially sensitive, and a large efflux

of malate is not expected. Additionally, malate is a weaker chelator than the citrate used in this experiment, requiring much higher concentrations for Al detoxification (Ryan *et al.*, 1995). Al chelation described here is therefore expected to be solely based on the presence of citrate. Al accumulation in the roots was 293 (± 10) μmol (g root DM)$^{-1}$ in the Al treatments and 178 (± 26) μmol g^{-1} in the Al/citrate treated plants. The latter Al concentration was surprisingly high, regarding the decrease in Al effects observed in the growth parameters. Equilibrium calculations with ECOSAT showed a decrease in solution activity of Al from 38 to 4 μM, when including 100 μM citrate in the nutrient solution with Al. A decrease in activity would generally mean less Al interaction with the apoplast binding sites and therefore less accumulation in the root. Though the Al concentration in the roots of Al/citrate treated plants is lower than in the Al treatment, it would be expected to have a larger effect on growth than the small decrease in root elongation and dry matter observed here. A similar accumulation and exposure time caused a 50% reduction in wheat root length in earlier experiments (chapter 4 of this thesis). The decreased FiNC for Cu, Zn, Mg and K in the roots are signs of the presence of Al, as is the still slightly increased FiNC for root P, though the effects are limited. There are some possible explanations for the limited effect of Al on growth and nutrient concentration at a relatively high root Al concentration, as shown with the Al/cit treatment. The first and most likely one is that the Al is mainly present as a harmless complex with citrate. Little interaction with the root would be expected when present as Al-citrate, since the complexation with an organic anion is generally considered to be the mechanism for excluding Al from the root. Nevertheless, in this case it appears to be bound in the root apoplast, since washing did not remove it from the root. Root P concentration in the Al/cit treatment was elevated compared to the control and part of the Al may also be present as Al-phosphate precipitate. A second possibility is that accumulation in the root did occur but at a lower rate than in the Al treatment. The low Al^{3+} activity in the presence of citrate allows only small amounts of Al to interact with the root at a given moment, causing less disturbance in root growth and nutrient uptake. Solution equilibrium will maintain a certain concentration of free Al^{3+} in the solution, slowly adding Al to the root surfaces.

Citrate strongly improved plant growth in the presence of Al, compared to the Al treatment. The major part of the Al-induced effects on dry matter production and root elongation was prevented and the observed decrease in root growth in the Al/cit treatment can not be distinguished from the effect of having only citrate in the nutrient solution.

The effect of citrate on Al toxicity is less absolute when it comes to nutrient uptake. Several nutrient concentrations in the root were below concentrations found in the control plants. Of these nutrients, Mg and K are most likely directly affected by the Al in the root, since citrate did not influence their uptake. Uptake of Cu, Zn and Fe is decreased or very variable in the citrate treatment and citrate may also have interfered in the Al/citrate treatment by decreasing the activity of free cations in the solution. However, in the case of root Cu and Zn concentrations, the effect is such that it cannot be explained by citrate influence alone and the larger part of the effect on these nutrients has to be ascribed to the accumulated Al in the root.

Citrate was effective in preserving wheat growth within the time limit of this experiment. However, the ratio citrate : Al may need to be substantially larger than 1 to fully prevent a decrease in nutrient concentration by Al. At the same time high citrate concentrations will influence uptake of the same nutrients. This seems to make the effect of citrate and thus also root citrate exudation as Al-resistance mechanism, somewhat dualistic.

7

Epilogue

In this final chapter the main results of the previous chapters are summarised and discussed in a more general context. First, the Al interference with plant nutrient uptake is addressed, with special emphasis on Al-specific effects on trace element uptake. In the second paragraph, the putative role of the cell wall in Al toxicity and plant nutrient uptake is discussed. Paragraph 3 briefly summarises the role of the pH in Al cell wall adsorption and in Al toxic effects on nutrient uptake. Al induced changes in plant growth are widely used and an easily measurable indicator for Al toxicity. A possible connection between Al accumulation and plant growth is discussed in §4, followed by the positive and some negative effects of Al complexation by organic chelators (§5). Finally, some points of interest for future research in Al toxicity are listed in §6.

§1. The influence of Al toxicity on nutrient uptake in the plant

Al decreased the uptake of the macronutrients Ca, Mg and K in tomato and wheat, which is a well-known symptom of Al toxicity. The experiments in the previous chapters show that Al also strongly affected the uptake of Cu, Zn and Mn. The inhibition of uptake of Cu and Zn was an immediate response to Al exposure and was not depending on the ambient pH. In the short-term experiment of chapter 6, Al even caused a net efflux of Cu and Zn from the root by allowing less Cu and Zn to enter the root than was translocated to the shoots. Trace elements are involved in many redox processes and enzyme activities in the plant and a deficiency in trace metals can lead to severe malfunction of plant metabolism (Clarkson and Hanson, 1980; Römheld and Marschner, 1991). Though the decrease in uptake was substantial, it is not clear to what extent deficiency actually occurred in the tomato and wheat roots and what effect a nutrient deficiency in roots could have had on plant functioning. A more detailed knowledge of nutrient deficiency levels in plant roots, including micronutrients, will help to distinguish primary and secondary effects of Al toxicity.

Uptake of cations is mediated by either specific cation transporters or through less selective cation channels, in which uptake is driven by the electrical membrane potential (Gaither and Eide, 2001; Hart *et al.*, 1998).

Since Al can interfere with ion channels, cause a depolarisation of the plasma membrane and compete as a cation with the nutrients for binding sites, both transport mechanisms can have been inhibited by the presence of Al. There is at present no knowledge of a specific interaction of Al with transporters for trace elements or for Mg (Fox and Guerinot, 1998; Gassman and Schroeder, 1994). Mg was especially sensitive to the Al in the solution and its uptake was already inhibited at the lowest Al activity when other nutrients were not affected. Overexpression of a Mg transport system in the membrane conferred Al resistance on *Saccharomyces cerevisiae*, indicating that in yeast the specific uptake of Mg over the cell membrane was a target for Al toxicity (MacDiarmid and Gardner, 1998). Al can specifically block Ca (-dependent) and K channels. Al interferes with K^+ channels in the plasma membrane, blocking both in- and outward directed K^+-fluxes (Gassmann and Schroeder, 1994; Liu and Luan, 2001). Additionally, Al-induced release of organic anions is usually accompanied by a K^+ efflux for electro-neutrality (Osawa and Matsumoto, 2002). The K concentration was generally more affected in tomato than in wheat, which is most likely caused by species differences in regulation of the membrane K-fluxes.

§2. Cation adsorption to the root cell wall; its possible role in uptake and Al toxicity

Nutrients adsorbed to the cell wall are generally considered to form an easily exchangeable metabolic pool of nutrients, available for uptake into the cell when needed. An exception is the adsorption of Ca, which is mainly present in the apoplast as a structural component of the cell wall. With respect to nutrient uptake, cell wall adsorption may be a mechanism for a plant to even out heterogeneity in nutrient availability. Nutrients would adsorb at times of high solution concentration, being released to the solution again at moments of deficiency because a new equilibrium establishes itself. Alternatively because the plant mobilises the nutrients by producing chelators, like organic acids or other phytometallophores (Welch, 1995). Especially on acid soils, which are poor in base cations, a mechanism to optimise nutrient availability may make a difference to plant survival. A mechanism for a relationship between cell wall adsorption and nutrient uptake is still elusive.

Keltjens (1995) showed a decrease in both Mg adsorption in the apoplast and Mg root uptake, with increasing Al concentrations in maize roots. Apoplast adsorption of Cu contributed to its uptake in ryegrass in the absence of Al (Thornton and Macklon, 1989) and the Al-induced decrease in cell wall adsorption in tomato may have contributed to the inhibition in uptake in our experiments. The uptake of K and Ca has a different position than the other cations in the discussion about the role of the cell wall in nutrient uptake. K adsorption to the cell wall is minimal and the K concentration in the root is in that sense not affected by Al, though Al often decreases uptake of K into the cell. Ca is the most abundant cation in the root cell wall, which forms its major storage compartment. Al strongly decreased Ca adsorption to the cell wall, yet the effect of Al on root Ca concentration in whole plant experiments is usually small to non-existing. How does this rhyme? Chemical analysis of the total root material will include all root compartments and even though the cell wall Ca concentration may have been as much affected as it was in the isolated cell wall material, the change may be obscured. In whole plant experiments, the cell wall surface may not have been so fully exposed to the Al in the solution as in adsorption experiments with isolated cell walls, leaving Ca adsorption relatively undisturbed in some parts. However, this argument would be equally valid for a cation like Cu, which does show a clear decrease in concentration. More likely candidates for maintaining a relatively high Ca concentration are the intracellular Ca storage compartments, like the vacuole and the endoplasmic reticulum. These may be not or to a lesser extent affected by Al, which will limit the change in total Ca concentration in the root (Marschner 1995; Zhang and Rengel, 1999). Al can cause an increase in cytosolic Ca concentration through inhibition of Ca efflux from the cell or by Ca release from intracellular compartments. Though significant for the cytoplasm, the increase will have hardly any effect on the overall Ca concentration of the root, since Ca concentration in the cell is kept very low and an increase in cytosolic Ca will be insignificant, compared to apoplast Ca concentration (Ma et al., 2002; Zhang and Rengel, 1999). Additionally, an increase in Ca concentration in the stele, as a result of Al exposure, has been reported, which may (partly) compensate in the root Ca concentration for loss of Ca from the cell wall (Jentschke *et al.*, 1991).

Finally, *in vitro* adsorption experiments will no doubt create a different environment for cell wall adsorption and discrepancies between our results and what happens *in vivo* may exist.

Aluminium strongly adsorbed to the cell wall binding sites of tomato roots, at the expense of the nutrient cations (chapter 3). The adsorbed Al fraction will not participate in cation competition for uptake or interfere with ion transporters, as long as the Al activity and the pH in the solution remain unchanged. However, with a decrease in Al activity or pH cell wall bound Al may desorb again and interfere with nutrient uptake. Al activity in the apoplast solution decreases, e.g. because of a plant-induced increase in apoplast pH or of heterogeneity of the soil. Apoplast pH may (locally) decrease as a result of proton efflux from the cells. With the high affinity of the cell wall for Al, relatively high H^+ concentrations would be needed to release Al from the cell wall (chapter 2). In both cases a new equilibrium will be established in the apoplast and the adsorbed fraction will supply the solution with extra Al^{3+}. The extent of Al ad/desorption will depend on the ratio between Al and proton activities in the solution. With the addition of Al^{3+} from the cell walls, the Al exposure of the apoplast may be more than would be expected on the basis of the Al concentration in the external solution.

A clear conclusion on a functional link between Al adsorption to the cell wall and nutrient uptake cannot be drawn from the results in this thesis. However, it can be concluded that in tomato the nutrients, which were most affected by Al in cell wall adsorption, were also most inhibited in uptake (Mg, Cu, Zn). Chelating Al with citrate or EDTA prevented Al adsorption to the cell wall and maintained adsorption of most nutrient cations. Under equivalent conditions, less Al accumulated in the plant roots and Al-induced inhibition of nutrient uptake was in most cases prevented. Though Cu and Zn uptake were often still affected, this was most likely due to a side effect of the chelator. A decrease in pH also strongly decreased Al adsorption to the cell wall and Al root accumulation. Despite the lower Al accumulation, nutrient uptake did not improve, since a high proton concentration can also interfere with nutrient uptake. Under these circumstances, nutrient cation adsorption was probably also decreased, like it was shown for Ca in chapter 2. An exception may be Cu adsorption, because of the high affinity of the cell

wall for Cu cations. The decrease in pH, which decreased Al accumulation, may have maintained Cu adsorption, which may have been the reason for the improvement of Cu uptake at low pH. A study of nutrient uptake with e.g. isotope tracers may lead to more conclusive evidence for a role of the cell wall in nutrient uptake.

§3. Al toxicity and pH

Al toxicity and pH in the plant apoplast are mutually dependent: the pH can influence Al speciation, the extent of Al adsorption to the cell wall and the strength of Al^{3+} in the competition for cation uptake, within certain pH ranges. Reversely, Al influences proton fluxes over the plasma membrane and with that the extent of acidification of the root apoplast. Al root accumulation may be seen as a reflection of the competition position of Al in the apoplast: at a low pH, less Al adsorbed and accumulated, since the protons formed a stronger competition. At the same time a decrease in pH increased the activity of free Al^{3+} in solution, which interfered with cation uptake. A low pH generally also meant more interference from H^+ in nutrient uptake, besides the Al^{3+} competition, and the effects of Al^{3+} and H^+ were synergistic, antagonistic or independent (chapter 2 and 5). It is difficult to distinguish between proton and Al toxicity, since both can lead to a decrease in nutrient uptake and root elongation. The clearest results for an Al effect without a direct influence of the pH on nutrient uptake were the concentrations of Cu and Zn. A decrease in pH did not affect the uptake of both nutrients but they were significantly decreased in the presence of Al. When an already low apoplast pH decreased even further, the high H^+ concentration prevented the Al effect on Cu and Zn uptake, which retained levels like found in the absence of Al.

§4. Al toxicity and plant growth

A larger CEC of the root cell walls, as found in dicots like tomato, can provide more binding sites for Al and form a larger exchangeable pool of Al. At least part of the Al bound to the cell wall may be directly related to root length inhibition, through interference with cell wall components and with enzymes that are involved in elongation. An increase in pectin content and therefore

in binding sites increased both Al accumulation and Al sensitivity in maize (Schmohl and Horst, 2000). Does this mean that tomato with its higher CEC was more sensitive to Al than wheat? A positive correlation between CEC and Al tolerance could not be found when a large range of plant genotypes was compared for CEC and Al tolerance (Horst, 1995). Tolerant plant species often accumulate less Al than sensitive ones but this is more based on tolerance mechanisms than on the number of cell wall binding sites (Barceló *et al.*, 1996). However, the Al accumulation in tomato roots was generally larger and at a higher rate. The inhibition in dry matter production and root elongation was also larger, though the effects varied with pH conditions. Yet, this is not enough to draw any conclusion on Al sensitivity of tomato or wheat. A linear correlation between Al accumulation in the plant root and inhibition of dry matter production is often difficult to establish. A plant's growth response to Al depends on its sensitivity to Al, its capacity to avoid the toxic effects of Al but also its sensitivity to nutrient deficiency. Additionally, a possible correlation at low Al exposure may become obscured at higher levels of Al accumulation, when a further increase in Al concentration in the root no longer has an additional toxic effect. The threshold value for Al accumulation at which maximal toxic effects occur will be relatively low for Al-sensitive plant species. The rate of accumulation also determined the Al effect: wheat accumulated Al in the root more slowly than tomato and the response in dry matter production showed a longer lag. Al-induced changes in nutrient uptake and dry matter production were initially uncoupled: nutrient uptake was inhibited before dry matter responded to Al. The change of the relative growth rate and the relative nutrient uptake rate in time in chapter 4 emphasises that conclusions drawn on the basis of short-term exposure may not be valid over a longer time period. Root elongation was effected immediately, like the uptake of some of the nutrients, and as such it is often used as a sensitive marker for Al toxicity. In the final experiment with Al and citrate, wheat root elongation was almost at control level and specific root length was identical to that in control plants (chapter 6). Still, the wheat plants had a relatively high Al concentration, a negative Cu influx in the roots and Zn, Ca and K concentrations in the root, which were marginal. The absence of growth inhibition clearly does not automatically mean that Al did not have any effect.

§5. The role and efficiency of chelators in Al toxicity

Citrate, present in an equal concentration to Al, was quite effective in reducing the Al effects on growth and on nutrient cell wall adsorption (chapter 2, 3 and 6). It decreased but did not prevent Al accumulation in the wheat roots in the final experiment, though the Al in the root had only limited effect on plant growth. Next to Al-citrate complexes, low concentrations of free Al^{3+} will have been present in the apoplast solution, because of protonation of citrate at the low pH, which made it not fully available for chelating Al. Citrate also chelated Cu and Fe in the solution, decreasing its solution activity and uptake in the plant. Cu adsorption to the cell wall was on average lower, though results were variable (chapter 3). Citrate did not affect Zn adsorption to the cell wall but the Zn uptake and translocation to the shoot in chapter 6 were different than found for control plants. Growth was maybe not limited within the limits of the last experiment but the inhibition in nutrient uptake would most likely have caused serious problems on the longer term. Higher citrate concentrations would be necessary to fully prevent Al toxicity but this will at the same time increase interference with trace element uptake. An Al tolerance mechanism in a plant, based on organic anion exudation, appears to solve one problem for the plant while creating the next.

The effect of malate on preventing Al adsorption was very limited, when present 1:1 with Al. This agrees with Al-malate being a less stable complex and with the observation that a much higher malate to Al ratio is needed to detoxify the Al (chapter 2; Ryan *et al.*, 1995). EDTA was equally effective in chelating Al as citrate was. It reduced the Al^{3+} activity in the solution and the Al cell wall adsorption even more than citrate did, when Al and chelator were present in equimolar concentration (chapter 4 and 5). Yet, EDTA had the experimental disadvantage of interfering strongly with Cu and Zn. Depending on the sensitivity of the plant species to reduced uptake of these trace elements and on the duration of the experiment, this will cause undesired side effects. Tomato seemed to be able to circumvent the problem at relatively higher pH; possibly it could release the nutrients from the EDTA-complex better than wheat could.

§6. Prospects in Al research

There are many facets of Al toxicity, which are still unresolved. One important aspect is the mechanism for uptake of Al over the plasma membrane. Once the pathway of Al into the cell is known, it may become clear how Al interferes with nutrient uptake. The effect of Al on the trace elements needs further attention, to understand their possible role in the plant's response to Al toxicity. One conclusion from the work presented here is that the pH plays an important role in Al toxicity, by changing its accumulation in the plant root and influencing nutrient uptake. More sophisticated methods for *in vivo* measurement of the apoplast pH are needed to interpret Al effects on nutrient uptake and cell wall functioning in more detail. Finally, the regulation of Al-tolerance is under intensive study and though the knowledge on internal and external detoxification of Al has increased over the past years, the full picture is not yet clear. Understanding the way a plant responds to unfavourable environmental conditions, like Al toxicity, or how it can tolerate them is of great importance for improving agriculture and understanding complex ecosystems on acid soils.

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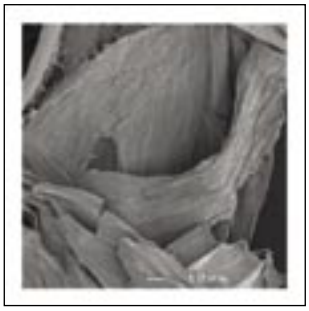

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Growing tomato plants in the greenhouse for isolation of cell wall material

Scanning Electron Microscope picture of dried cell wall material at 650x magnification

The difference in growth of tomato plants after 3 weeks of exposure to 0 or 100 µM Al at pH 4

Inhibited development of lateral roots in tomato, as a result of Al exposure (20x magnification)

Set-up for the nutrient film technique in the climate controlled room

Aluminium toxicity is a serious problem for many plants growing on acid soils. Plant root elongation and nutrient uptake are inhibited and overall plant growth is poor. The extent of the Al effect will depend on (1) the activity in the soil solution of free Al^{3+}, the most phytotoxic aluminium species and (2) the capacity of a plant to tolerate both high Al and high proton concentrations in the rhizosphere. The Al^{3+} activity in the solution is itself a resultant of the total Al concentration, pH, the activities of other cations and the presence of soil organic matter, which can bind Al. Many of the more Al-tolerant plants can produce high concentrations of Al-chelators, like the low molecular weight (LMW) organic anions citrate and malate. Al-organic anion complexes formed in this way are either excluded from entering the plant cells (external detoxification) or transported to and stored in designated plant parts, like the leaves (internal detoxification). Complexation will take place in the rhizosphere or in the root apoplast, which is the plant compartment outside the plasmalemma, consisting of cell walls, cell wall pores, intracellular spaces and the outer surface of the plasma membrane. Local pH changes can influence the Al effect on the plant but it may also vary the complexation by organic anions. There are still many questions and uncertainties about the interaction of Al or organo-Al complexes with the plant. The intertwinement of effects of Al, organic anions and pH, resulting in a more or less Al-affected plant, was the motivation for starting this project.

What is the fate of these organo-Al complexes in the root? Do they interact with the root apoplast and particularly with the cell wall?

Pectins in the root cell wall have a high capacity to bind cations, nutrients as well as Al, and these cell wall polymers can accumulate Al in high concentrations. We investigated the interaction of Al with root cell walls and the competition of Al with other cations for cell wall binding by performing *in vitro* adsorption experiments on isolated root cell walls from tomato. Tomato is used as a representative of the dicotyledonous plants, which have a higher pectin content and cation binding capacity than the monocotyledons.

Ca is the main cation to adsorb to cell wall binding sites under Al-free conditions. There it forms among others cross-links between the pectic chains.

Al replacement of Ca at the cell wall binding sites increases the rigidity of the root and the presence of Al in the root disturbs Ca homeostasis. How exactly Al induces inhibition of root elongation still needs to be clarified. Al formed a strong competitor for the adsorption sites in the experiments and Ca adsorption to the cell wall was almost eliminated in the presence of Al. Protons in their turn strongly decreased Al and Ca adsorption with decreasing pH (chapter 2). The competition between Al^{3+}, Ca^{2+} and H^{+} for adsorption sites could be very well described with a Gaines-Thomas exchange model, with the carboxylic groups of pectin as the surface binding sites. Some results indicated that additional sites were involved in the adsorption of Al (but not of Ca) for which a heterogeneous adsorption model would be more suitable. We used the organic anions citrate and malate to study the effect of these plant-own chelators on Al interaction with the cell wall. Addition of citrate could prevent most of the Al adsorption and maintained Ca adsorption near its control (-Al) level. The weaker chelator malate had only a limited effect on Al adsorption.

Which role can Al adsorption to the cell wall play in inhibition of nutrient uptake?

In chapter 3, we approach the Al competition for adsorption to the cell wall from a biologically more realistic point of view with the use of a full nutrient solution, the micronutrients inclusive, for cell wall adsorption experiments with Al and nutrient cations. Adsorption of nutrient cations, like Mg, Cu and Zn, to the pectic binding sites may positively influence their uptake, creating a pool of readily exchangeable nutrients in the apoplast. How large or how essential this influence is, still remains to be elucidated. Assuming that cell wall adsorption makes a difference, the competition of Al for the binding sites would add to the inhibition of nutrient uptake.

Al adsorbed strongly to the cell wall, like in chapter 2, while significantly decreasing the adsorption of Ca, Mg, Zn and Cu. Citrate and ethylenediamine tetraacetic acid (EDTA) were used as Al-chelators, the latter as a less easily degradable replacement for citrate. Both chelators could prevent Al from competing with the nutrient cations for adsorption, though this effect was only complete when Al activity in the solution had been reduced by 90%.

Using a range of chelator concentrations, we created different concentrations of Al on the cell wall surface. The decrease in Ca, Mg, Zn and Mn adsorption was linearly and negatively related to increasing Al adsorption. This could not be made visible for Cu, due to the strong interaction of the chelators with this micronutrient.

Do organo-Al complexes in the rhizosphere or in the root apoplast interfere in any way with plant growth and nutrient uptake or are they fully inert in that respect? How efficient and absolute is the counteracting of Al toxicity by organic anions and what are the prerequisites for such an Al-detoxification?

The following three chapters describe the effects of Al and Al-organic anion complexes under different circumstances, in whole plant hydroponic experiments. Besides tomato we used wheat, representing in these the monocots with their lower pectin content in the root cell walls. The experiments in chapter 4 and 5, which extended over more than one week, were performed with EDTA as Al-chelator because of the rapid microbial breakdown of citrate in solution. Citrate as chelator returns in chapter 6.

Al accumulation, plant growth and nutrient uptake responses to Al (-EDTA) exposure were monitored in tomato and wheat seedlings over a period of 3 weeks (chapter 4). Wheat accumulated Al much more slowly than tomato, which could be related to the lower binding capacity of the wheat root cell wall. Al caused a decrease in relative growth rate but not until days after the relative uptake rate of most nutrients had been reduced. Al strongly decreased Cu and Zn uptake in both plant species, an effect, which could be seen from the first day of Al exposure. It also induced Mg deficiency but the Ca concentration, which is a usually affected in Al toxicity, did not become deficient within the time period of this experiment. Addition of EDTA could prevent Al inhibition of growth and nutrient uptake in tomato but wheat growth was severely reduced at the highest EDTA concentration.

Plants growing on acid soils can be exposed to toxic concentrations of Al but also of protons. Since both can inhibit (root) growth and nutrient uptake, it is often very difficult to distinguish between Al and pH effects on plants. Yet, this distinction is important for a full understanding of specific

phytotoxic effects of Al. Chapter 5 describes the effect the ambient pH had on Al toxicity, plant growth and nutrient uptake and on the effect of EDTA. Tomato and wheat plants were exposed to Al at 2 different pH levels in the nutrient solution, in the presence or absence of the chelator. In a second experiment the plants themselves created a change in apoplast pH as a result of differences in N-nutrition and its effect on Al toxicity was evaluated.

Plant growth generally responded strongly to Al and only little to a lower pH or NH_4 in the solution. Growth improvement by chelating Al with EDTA was very limited. Al induced a specific decrease in Cu and Zn uptake and translocation in wheat, which was not affected by relatively small pH changes. However, the Al-induced decrease could be (partially) prevented with NH_4-nutrition, an effect, which is presumably caused by strong apoplast acidification following NH_4-assimilation. The response in Mg concentration in wheat shoots was most likely pH independent and Al specific, whereas Ca and K concentrations did respond to the pH but only in the presence of Al. Nutrient uptake in tomato was generally sensitive to both low pH and Al, which made it very difficult to indicate precisely what the Al effect had been. The use of an Al-chelator, like Na_2-EDTA, was effective in reducing Al-induced effects on uptake of most nutrients but EDTA interfered with Cu and Zn uptake.

Citrate is one of the major LMW organic anions that Al-tolerant plants can exude for chelating and detoxifying toxic Al. To study the effect of this organo-Al complex on nutrient uptake, we exposed wheat seedlings for 110 hrs to Al(-citrate). For this and to prevent breakdown of the citrate in solution, we used an adaptation of a nutrient film technique, which constantly supplies the plants with fresh solution instead of recirculating it (chapter 6).

Citrate allowed Al to accumulate to a surprisingly high concentration in the wheat roots, probably as an Al-citrate complex. The citrate in the solution did prevent the Al-induced decrease in root dry matter production and root elongation. It did not, however, accomplish to maintain full nutrient uptake and net root uptake of Mg, Cu, Zn and Fe were still reduced. Whereas the accumulated Al in the root most likely affected uptake of Mg, Cu and Zn, the high citrate concentration in the solution appeared to influence the uptake of Cu, Zn and Fe. This would mean a possible negative side effect of citrate exudation if active as an Al tolerance mechanism.

In the epilogue (chapter 7) we look back on the previous chapters and place the results in a broader perspective. Chelators and a low pH were both effective in preventing Al binding to the cell wall and in restoring nutrient adsorption. The effect of the chelators for a large part extended to the plant growth experiments: less Al accumulated and growth and nutrient uptake improved, compared to Al treated plants. High proton concentrations also reduced Al accumulation in the living root but it did not improve nutrient uptake. More precise data on apoplast pH and localisation of Al and nutrients in the apoplast are needed to answer the questions on the role of the cell wall in plant nutrition.

So far there has been little attention in literature for the effect of Al or organo-Al complexes on micronutrient uptake. The results in this thesis show that Al severely decreased especially Cu and Zn uptake and their adsorption to the cell wall. A low pH and subsequent decrease in Al accumulation improved uptake of these two micronutrients, indicating a possible direct relationship between Al cell wall adsorption and uptake of Cu and Zn. Yet adsorption and uptake of especially the Al-affected micronutrients were inhibited by the Al-chelators, creating a conflict in positive and negative effects of organic chelators in Al toxicity.

Aluminium toxiciteit is een groot probleem voor veel planten op zure bodems. In deze planten zijn de wortelstrekking en nutriëntenopname geremd en de planten groeien over het algemeen zeer matig. De grootte van het Al effect hangt af van (1) de activiteit van vrij Al^{3+}, de meest phytotoxische aluminium species, in de bodemoplossing en (2) de capaciteit van een plant om hoge concentraties van zowel Al als protonen in de rhizosfeer te tolereren. De Al^{3+} activiteit is zelf een resultante van de totale Al concentratie, de pH, de activiteit van andere kationen en de aanwezigheid van Al-bindende organische stof in de bodem. Veel Al-tolerante planten kunnen hoge concentraties Al chelatoren produceren, zoals de organische anionen citraat en malaat. De zo gevormde Al-organisch anion complexen worden òf niet of nauwelijks opgenomen in de wortelcellen (externe detoxificatie), òf ze worden wel opgenomen en vervolgens getransporteerd en opgeslagen in specifieke delen van de plant, zoals de bladeren (interne detoxificatie). Complexering vindt plaats in de rhizosfeer of in de wortel-apoplast, het compartiment in de plant dat zich buiten de plasmalemma bevindt en dat bestaat uit celwanden, -poriën, intracellulaire ruimtes en het buitenoppervlak van de plasmamembraan. Lokale wisselingen in pH kunnen zowel het Al effect als de complexering door organische anionen beïnvloeden. Er bestaat nog steeds veel onduidelijkheid over de interactie tussen Al of organische Al-complexen en een plant. De verstrengeling van effecten van Al, organische anionen en pH, resulterend in een meer of minder door Al aangetaste plant, was de motivatie voor de start van dit project.

Wat is het lot van deze organische Al-complexen in de wortel? Vertonen ze enige interactie met de wortel-apoplast en in het bijzonder met de celwand?

Pectine in de wortelcelwand heeft een hoge capaciteit voor het binden van kationen, zowel nutriënten als Al, en deze celwandpolymeer kan hoge concentraties Al accumuleren. Wij onderzochten de interactie tussen Al en wortelcelwanden en de competitie tussen Al en andere kationen voor het binden aan de celwand door het uitvoeren van *in vitro* adsorptie experimenten met geïsoleerde wortelcelwanden van tomaat. Tomaat is gebruikt als een vertegenwoordiger van de dicotylen, die een hoger pectinegehalte en een

grotere kation bindingscapaciteit hebben dan de monocotylen. Onder Al-vrije omstandigheden worden de meeste bindingsplaatsen aan de celwand bezet door calcium, dat o.a. crosslinks vormt tussen de pectineketens. Vervanging van Ca aan de celwand door Al verhoogt de rigiditeit van de wortel en de aanwezigheid van Al in de wortel verstoort de Ca homeostase. Het exacte mechanisme voor Al-geïnduceerde remming van de wortelstrekking moet nog opgehelderd worden. Al vormde in de experimenten een sterke concurrent voor de bindingsplaatsen en Ca adsorptie aan de celwand was in hoge mate gehinderd door de aanwezigheid van Al. Protonen op hun beurt verlaagden zowel Al als Ca adsorptie bij dalende pH (hoofdstuk 2). De competitie tussen Al^{3+}, Ca^{2+} en H^{+} voor bindingsplaatsen kon heel goed beschreven worden met een Gaines-Thomas omwisselmodel, met de carboxylgroepen van pectine als de bindingsplaats aan het oppervlak. Sommige resultaten wezen op de betrokkenheid van andere bindingsplaatsen bij de adsorptie van Al (maar niet van Ca), waarbij een heterogeen adsorptie-model gebruikt zou moeten worden. Wij maakten gebruik van de organische anionen citraat en malaat om het effect van deze plant-eigen chelatoren op Al interactie met de celwand te bestuderen. Toevoeging van citraat kon Al adsorptie grotendeels voorkomen en hield Ca adsorptie bijna op het controle-(–Al) niveau. De zwakkere chelator malaat had maar een beperkt effect op Al adsorptie.

Welke rol kan Al adsorptie aan de celwand spelen in de remming van nutriëntenopname?

In hoofdstuk 3 benaderen we de Al competitie voor celwand adsorptie vanuit een biologisch realistischer perspectief door een volledige voedingsoplossing te gebruiken, inclusief sporenelementen, in celwand adsorptie experimenten met Al en nutriënt kationen. Adsorptie van nutriënt kationen, zoals Mg, Cu en Zn, aan pectine kan mogelijk een bijdrage leveren aan hun opname door het vormen van een gemakkelijk uitwisselbare pool aan nutriënten in de apoplast. Hoe groot en hoe essentieel deze bijdrage is, is nog niet duidelijk. Als we aannemen dat celwand adsorptie uitmaakt voor nutriëntenopname, dan kan de competitie van Al voor de bindingsplaatsen bijdragen tot de remming van de nutriëntenopname. Al adsorbeerde sterk aan de celwand, zoals in hoofdstuk 2, en veroorzaakte een significante vermindering in Ca, Mg, Zn en Cu adsorptie.

Citraat en ethylenediamine tetraacetic acid (EDTA) werden gebruikt als Al-chelatoren, de laatste als een minder gemakkelijk afbreekbaar alternatief voor citraat. Beide chelatoren konden Al weerhouden van competitie met de nutriënt kationen, hoewel het effect pas compleet was wanneer Al activiteit in de oplossing met 90% was verminderd. Door een reeks aan chelator concentraties te gebruiken, creëerden we verschillende Al concentraties aan de celwand. De afname in Ca, Mg, Zn en Mn adsorptie was negatief lineair gerelateerd aan de toename in Al adsorptie. Dit kon helaas niet zichtbaar gemaakt worden voor Cu, vanwege een sterke interactie van dit sporenelement met de chelatoren.

Belemmeren Al-organische complexen in de rhizosfeer of in de wortel-apoplast plantengroei en nutriëntenopname op enigerlei wijze of zijn ze volledig inert wat dat betreft? Hoe effectief en absoluut is het voorkomen van Al toxiciteit door organische anionen en wat zijn de voorwaarden voor zo'n detoxificatie?

De volgende drie hoofdstukken beschrijven de effecten van Al en organische Al-complexen onder verschillende omstandigheden, in een reeks watercultuur-experimenten met hele planten. Behalve tomaat hebben wij ook tarwe gebruikt, als vertegenwoordiger van de monocotylen met hun lagere pectinegehalte in de wortelcelwanden. De experimenten in hoofdstuk 4 en 5, welke meer dan 1 week duurden, werden uitgevoerd met EDTA als Al-chelator, vanwege de snelle microbiële afbraak van citraat in oplossing. Citraat als chelator keert terug in hoofdstuk 6.

Al accumulatie, plantengroei en nutriëntenopname als respons op blootstelling aan Al(-EDTA) werden gevolgd in tomaat en tarwe zaailingen gedurende een periode van 3 weken (hoofdstuk 4). Tarwe accumuleerde Al veel langzamer dan tomaat, iets wat gerelateerd kan zijn aan de lagere bindingscapaciteit van de celwanden van de tarwewortel. Al veroorzaakte een afname in relatieve groeisnelheid, maar pas enkele dagen nadat de relatieve opnamesnelheid van de meeste nutriënten was verlaagd. Al verminderde de opname van Cu en Zn sterk in beide plantensoorten, een effect dat al zichtbaar was na een dag blootstelling aan Al. Het veroorzaakte ook Mg gebrek, maar Ca deficiëntie, een veel voorkomend symptoom van Al toxiciteit, trad niet op binnen de tijdsspanne van dit experiment.

Toevoeging van EDTA kon Al-geïnduceerde remming van groei en nutriëntenopname voorkomen in tomaat, maar de groei van tarwe was ook bij de hoogste concentratie EDTA sterk geremd.

Planten op zure bodems kunnen blootgesteld worden aan toxische concentraties aan Al, maar ook aan protonen. Aangezien beide kationen de planten(wortel)groei en nutriëntenopname kunnen remmen, is het vaak heel moeilijk om onderscheid te maken tussen Al en pH effecten op planten. Dit onderscheid is echter van belang voor een volledig begrip van de specifieke phytotoxische effecten van Al. Hoofdstuk 5 beschrijft het effect dat de pH in de omgeving had op Al toxiciteit, plantengroei en nutriëntenopname en op het effect van EDTA. Tomaten- en tarweplanten werden blootgesteld aan Al bij 2 verschillende pH niveaus in de voedingsoplossing, met of zonder chelator. In een tweede experiment veroorzaakten de planten zelf een verschil in apoplast pH, als gevolg van verschillende stikstofbronnen, en het effect op Al toxiciteit werd bestudeerd.

Plantengroei reageerde over het algemeen sterk op het Al en maar weinig op de lagere pH of NH_4 in de voedingsoplossing. Groei verbeterde slechts in beperkte mate met de complexering van Al door EDTA. Al veroorzaakte en specifieke verlaging van opname en translocatie van Cu en Zn in tarwe. Deze verlaging was niet gevoelig voor relatief kleine veranderingen in de pH, maar kon wel (gedeeltelijk) worden voorkomen wanneer NH_4 werd gebruikt als stikstofbron, waarschijnlijk als gevolg van een sterkere verzuring van de apoplast na NH_4 assimilatie. De respons van de Mg concentratie in de tarwespruit was naar alle waarschijnlijkheid pH onafhankelijk en Al specifiek, terwijl Ca en K concentraties alleen verlaagd werden door een lage pH in de aanwezigheid van Al. Nutriëntenopname in tomaat was over het algemeen gevoelig voor zowel pH als Al, wat het heel moeilijk maakte om precies aan te geven wat het Al effect was. EDTA als Al-chelator was effectief in het reduceren van Al-effecten op opname van de meeste nutriënten, maar het verstoorde de opname van Cu en Zn. Citraat is een van de meest voorkomende, relatief kleine organische anionen (Low Molecular Weight organic anions), die een Al-tolerante plant kan uitscheiden voor de complexering en detoxificatie van giftig Al.

Om het effect van deze organische Al-complexen te bestuderen, stelden wij tarwe zaailingen gedurende 110 uur bloot aan Al(-citraat). Om de afbraak van citraat in voedingsoplossing te voorkomen gebruikten wij in dit experiment een variatie op een "nutrient film technique", waarbij voortdurend verse oplossing langs de plantenwortels stroomde (hoofdstuk 6).

In de aanwezigheid van citraat accumuleerde verrassend veel Al in de plantenwortels, naar verwachting in de vorm van Al-citraat complexen. Het citraat voorkwam een Al-geïnduceerde remming van wortel droge stof productie en -strekking. Het kon echter niet de nutriëntenopname volledig op een controle niveau (–Al) houden en de netto opname van Mg, Cu, Zn en Fe waren nog steeds geremd. Het geaccumuleerde Al veroorzaakte naar alle waarschijnlijkheid de remming in opname van Mg, Cu en Zn. Hoge citraat concentraties leken ook de opname van Cu, Zn en Fe te beïnvloeden, wat een mogelijk negatief bijeffect van citraat exudatie bij Al-tolerantie zou kunnen betekenen.

In de epiloog (hoofdstuk 7) kijken we terug op de voorgaande hoofdstukken en plaatsen de resultaten in een breder perspectief. Chelatoren en een lage pH waren beide effectief in het voorkomen van Al binding aan de celwand en in het instandhouden van nutriëntenadsorptie. Het effect van de chelatoren was ook zichtbaar in de plantenexperimenten: minder Al accumuleerde en groei en nutriëntenopname verbeterden, vergeleken met Al-behandelde planten. Hoge proton concentraties verminderde ook Al accumulatie in de levende wortel maar verbeterde niet de opname van nutriënten. Meer exacte data voor apoplast pH en lokalisatie van Al en nutriënten in de apoplast zijn nodig om een antwoord te kunnen geven op de vraag welke rol de celwand adsorptie speelt in plantenvoeding.

Tot nu toe is er weinig aandacht geweest voor het effect van Al of organische Al-complexen op de opname van sporenelementen. De resultaten in dit proefschrift laten zien dat Al vooral een sterk remmend effect heeft op Cu en Zn opname en hun adsorptie aan de celwand. Een lage pH en als gevolg daarvan een lagere Al accumulatie verbeterde de opname van deze twee sporenelementen, wat een mogelijke aanwijzing kan zijn voor een verband tussen Al adsorptie aan de celwand en de opname van Cu en Zn.

Het feit dat juist de adsorptie en opname van de sporenelementen, die sterk worden beïnvloed door Al, ook geremd worden door een chelator als citraat, veroorzaakt een conflict tussen de positieve en negatieve effecten van organische complexeerders in Al toxiciteit.

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Met de verdediging van mijn proefschrift sluit ik mijn tijd als AIO af. Klaar! Op naar het volgende hoofdstuk. Hoewel er altijd momenten zijn in zo'n lang project dat het niet helemaal meezit, klimaatkamers die eindeloos buiten bedrijf waren, zaailingen die een geheimzinnige, vroegtijdige dood stierven nog voordat ik aan een proef was begonnen, zijn het jaren waar ik met veel plezier op terug kijk. Je leert veel in zo'n tijd en geleidelijk aan zie je jezelf groeien in je project. Je wordt kritischer en creatiever, in het onderzoek zelf, maar ook in de sociale aspecten van het onderzoeker zijn, het samenwerken en organiseren. Wat eens te meer is gebleken: onderzoek doe je niet alleen. Daarom wil ik, voordat ik dit boekje afsluit, een aantal mensen speciaal vermelden, die hebben bijgedragen aan het tot een goed einde brengen van mijn project.

Op de eerste plaats Willem Keltjens, mijn co-promotor en dagelijks begeleider, die er altijd voor me was als ik raad of commentaar nodig had. Willem, dankjewel voor je nimmer aflatende positieve houding en je bemoedigende woorden. Zelfs in deze laatste periode van mijn project, waarin voor jou het boek van Wageningen Universiteit al gesloten was, was je er voor je AIO's. Ik waardeer dit heel erg en ik zal onze lange gesprekken, over aluminium, maar ook over reizen, tuinen en allerlei andere zaken zeker gaan missen in de toekomst.

Ten tweede wil ik mijn promotor Willem van Riemsdijk bedanken voor de prettige en stimulerende samenwerking. De laatste maanden hebben we vooral via de e-mail moeten discussiëren, wat wonderwel goed ging, mede dankzij jouw snelle en uitgebreide reacties op de stukken die ik je toestuurde. Jouw kennis en expertise is het best merkbaar in de eerste twee artikelen en ik ben heel blij met datgene wat onze gezamenlijke inspanning opgeleverd heeft voor hoofdstuk twee.

Al mijn experimenten zijn tot stand gekomen in en rond het lab van Bodemvruchtbaarheid. En dat zou niet gelukt zijn zonder de praktische ondersteuning en adviezen van verschillende mensen. Op de eerste plaats Jaap Nelemans en Willeke van Tintelen. Jaap, ik weet dat je me af en toe nogal eigenwijs vond, maar ik weet ook dat ik altijd bij je terecht kon om te putten uit je jarenlange experimentele ervaring. Willeke en ik hebben gezellige uren doorgebracht in de zuurkast om tomatenwortels te "pletten" voor celwand materiaal. Bedankt voor jullie hulp!

De grotere plantproeven werden in de kas uitgevoerd en daarin werd ik ook bijgestaan door Arie Brader, Willem Menkveld en Peter Pellen. Dat was altijd gezellig en dankzij jullie kon ik er op vertrouwen dat mijn plantjes goed verzorgd werden op momenten dat ik er zelf niet kon zijn. Met Arie en Peter heb ik ook met veel plezier samengewerkt voor WEPAL, wat me na het aflopen van mijn AIO contract de kans gaf nog een tijd door te werken aan mijn proefschrift.

Nadat de planten geoogst waren moest er natuurlijk het een en ander gemeten worden en daarvoor had ik de expertise van de mensen van het Centraal Lab nodig: Arie van den Berg, Monique Driessen, Peter Nobels en in eerdere jaren ook Gerdine Gaikhorst en Erik Hey wil ik bedanken voor de ICP-AES, -MS en andere metingen in vele monsters plantmateriaal en voedingsoplossing.

Van de bovenverdieping zak ik weer een etage af, waar ik Ellis Hoffland wil bedanken voor haar bereidwilligheid om mijn stukken door te lezen en stevig te becommentariëren.

Het diepste punt van het gebouw vormde in veel opzichten meer een hoogtepunt: de onvolprezen "kelder" van de AIO's en Ph.D. studenten. Een plek waar je gemakkelijk bij elkaar binnen liep voor advies of als je je frustratie kwijt moest. Maar vooral een gezellige, hechte club mensen die elkaar ook buiten het werk wisten te vinden voor zeilweekends, pannenkoeken- of erwtensoep-avonden en wandeltochten. Ik wil speciaal Henriëtte, Dirkje, Anke, Renske, Annemieke, Lousie, Nicole en Jeanine bedanken voor de vriendschap, die gelukkig is blijven bestaan ondanks dat de meesten van ons inmiddels ergens anders een plek hebben gekregen.

Two more persons in the basement I especially would like to mention: Eduardo, we have shared an "office" and discussed aluminium and citrate over the past four years. You were the best roommate I could have wished for! Thank you for your friendship and help with the statistics and I wish you and Isabela lots of success for your future, back home in Brazil. Liping, thank you for initiating me in the wonderous world of fitting adsorption data.

Some of the data on cell wall binding in chapter 2 came from the work of Nell Romanova, who at one time worked as a post-doc at the Laboratory of Physical Chemistry and Colloid Science in Wageningen.

Nell, it was fun working with you and I hope I will have a chance one day to come and visit you in St. Petersburg!

Mijn project viel onder de C.T. de Wit Onderzoekschool voor Production Ecology and Resource Conservation, oftewel PE&RC. Mijn jaren in de AIO-raad en de onderzoeks- en onderwijscommissies van PE&RC heeft me veel geleerd over de ins en outs van onderzoeksprojecten en van een onderzoekschool, en over het organiseren van symposia. Alle PSP-ers: bedankt voor de samenwerking en de gezelligheid!

One of the last persons to help me finish my project is Magnus Burén, who put many hours of work in helping me with the (In)design of my thesis. Magnus, thank you so much!

En dan zijn er uiteraard de vrienden en familie buiten de universiteit, zonder wie ik mijn werk nooit had kunnen doen. Liesbeth en Magda, mijn paranimfen, alvast bedankt voor de morele steun bij de verdediging en alle keren daarvoor. Astrid voor onze ernstig-vrolijke discussies over het AIO zijn. Jet voor het bespreken van de kosmos. Marlies, mijn bezoekjes aan IJsselstein waren een oase van rust voor me. Mijn ouders kan ik helaas niet meer bedanken, maar ze zullen altijd een deel zijn van wie ik ben en wat ik doe.

Allan, tack så hemskt mycket för att du i alla avseenden har givit mig utrymmet och tiden att färdigställa min avhandling.

Jacqueline

Curriculum Vitae

Jacqueline (Jacqueline Wilhmelmina Maria) Postma werd op 10 december 1960 geboren te Oss. Na het behalen van het Atheneum B diploma in 1979, aan het Maasland College in Oss, besloot zij verder te gaan in wat al sinds haar vroegste jeugd haar grote hobby was: pianospelen. In 1980 werd zij toegelaten tot het voorbereidende jaar en een jaar later tot de docenten-opleiding aan het Utrechts Conservatorium in Utrecht. Wegens een armblessure moest zij de studie helaas na een aantal jaren afbreken.

In 1987 begon zij met de part-time studie Biologie aan de Rijksuniversiteit Utrecht. Deze studie, in een kleine, goedgemotiveerde groep studenten, bleek een schot in de roos. Omdat het part-time onderwijs niet alle onderwerpen kon bieden die haar interesseerden, stapte zij voor de laatste jaren over naar de full-time studie. In die periode deed zij ook haar twee afstudeerprojecten: ten eerste een jaar onderzoek aan wortelspecifieke transcriptie-factoren in zandraket bij de groep van Ben Scheres, vakgroep Moleculaire Genetica van de Universiteit Utrecht. Daarop volgde een halfjaar onderzoek aan cadmium toxiciteit en de inductie van phytochelatines in mais bij Willem Keltjens, vakgroep Bodemkunde en Plantenvoeding, Landbouw Universiteit Wageningen.

Toen vlak na haar afstuderen in 1995 een AIO-plaats beschikbaar kwam bij deze laatste groep, inmiddels beter bekend als de sectie Bodemkwaliteit van Wageningen Universiteit, greep zij deze kans met beide handen aan. Naast het onderzoek is zij ook jaren actief geweest in het Ph.D. Student Platform van de onderzoekschool Production Ecology and Resource Conservation. Haar project omtrent de phytotoxiciteit van aluminium(-organische complexen), met tomaat en tarwe als modelplant, begon in juli 1996 en eindigt met het gereedkomen en de verdediging van het proefschrift zoals u het nu in de hand heeft.

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