

**The role of silicon, boron and pH-dependent
aluminium speciation in solution on
aluminium toxicity in maize (*Zea mays* L.)**

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ABSTRACT

The toxic and inhibitive effects of aluminium (Al) on the growth and development of plants are well known, but the mechanisms of Al toxicity are not well understood, particularly the relative importance of symplastic versus apoplastic lesions of Al toxicity remains a matter of debate.

In agricultural practice, rectifying Al toxicity needs convenient and economic methods. In addition to liming, organic manure or phosphorous fertilizer application, silicon (Si) and boron (B) supply were suggested to alleviate Al toxicity, but results are controversial, especially the possible mechanisms of Al/Si and Al/B interactions have not been conclusively examined. Al toxicity occurring in high pH medium has been reported, but the mechanisms have not yet been clarified. There is even no consensus on which Al species are responsible for Al toxicity.

In this work, the role of Si, B and pH-dependent Al speciation in solution on Al toxicity was studied in an Al-sensitive maize cultivar Lixis. The main results are summarized below:

(1) Si treatment but not Si pre-treatment ameliorated Al-induced root injury as revealed by less root-growth inhibition and callose formation. Si treatment did not affect monomeric Al concentration in the nutrient solution suggesting an in-planta effect of Si on Al resistance. A fractionated analysis of Si and Al in the 1 cm root apices revealed that more than 85% of the root-tip Al was bound in the cell wall. Al contents in the apoplastic sap, the symplastic sap and the cell wall did not differ between -Si and +Si plants. Si did not affect the Al-induced exudation of organic acid anions and phenols from the root apices. However, Al treatment greatly enhanced Si accumulation in the cell wall fraction reducing the mobility of apoplastic Al. These results indicate that Si treatment leads to the formation of hydroxyaluminiumsilicates (HAS) in the apoplast of the root apex thus detoxifying Al.

(2) Based on the performance of root growth and callose formation, no evidence was found for an alleviative effect of B on Al toxicity. Various B supplies also did

not affect the Al content in the root tip. The B content in the root tip was only increased at very high B supply and was not influenced by Al treatment. The documented B/Al interaction might be due to the interaction of B and Al in the pectin network. Therefore, it was concluded that due to the low pectin content of grasses the overall effect of B is too weak to have any significant influence on Al toxicity in grasses.

(3) Aluminium reduced root growth to similar levels in solutions adjusted and maintained at pH 8.0 or pH 4.3 although the monomeric Al concentration of solution at pH 8.0 was four times lower than at pH 4.3. After 12 hours of Al treatment, Al contents of the 1 cm root apices of plants grown in solution at pH 8.0 was much higher than that at pH 4.3. However, Al-induced callose formation in the root apices was marginal and root-tissue integrity was better maintained at pH 8.0 than at pH 4.3. The largest fraction of the root-tip Al was recovered in the cell-wall fraction independent of the culture-solution pH. A lower percentage of Al was recovered in the acid-wash and base-wash solutions but a higher percentage in the symplastic sap fraction in the root tips grown at alkaline pH. A sequential extraction of the isolated cell-wall material with increasing KOH concentrations suggests that most of the cell-wall Al was precipitated $\text{Al}(\text{OH})_3$ in root tips exposed to Al at pH 8.0. This can be explained by a drastic pH reduction in the root apoplastic sap at bulk solution pH 8.0. These results can be interpreted as circumstantial evidence that at bulk solution pH 8.0 the maintenance of an acidic apoplast leads to the formation of cationic Al hydroxyl species and $\text{Al}(\text{OH})_3$ inducing root-growth inhibition but less plasma-membrane and cell damage than Al^{3+} dominating at pH 4.3.

The results presented demonstrate that Al in the root is mainly localized in the apoplast of the root apex. Different Al species in the root apoplast are not equally toxic. Highly positively charged mobile Al^{3+} is more effective in inducing callose formation and reducing cell integrity and root growth. The formation of HAS and of less positively charged hydroxyl-Al species in the apoplast reduces Al toxicity.

Keywords: Aluminium toxicity, apoplast, maize

ZUSAMMENFASSUNG

Der toxische und hemmende Einfluß von Aluminium (Al) auf Wachstum und Entwicklung der Pflanzen sind gut dokumentiert, aber die Mechanismen, die den Reaktionen zu Grunde liegen sind noch nicht bekannt. Dies gilt insbesondere für die Frage, ob primär symplastische oder apoplastische Läsionen für die Ausprägung von Al-Toxizität von Bedeutung sind.

In der landwirtschaftlichen Praxis werden einfache und ökonomisch günstige Methoden benötigt, um Al-Toxizität zu vermindern. Daher wird neben den bereits angewandten Methoden wie Kalkung, organische Düngung und Phosphatdüngung eine Anwendung von Silizium (Si) und Bor (B) diskutiert. Die Ergebnisse zum Einfluß von Si und B auf die Verminderung der Al-Toxizität sind widersprüchlich. Über den Mechanismus, der eine mögliche Interaktion Al/Si und Al/B erklären könnte, liegen noch keine gesicherten Erkenntnisse vor. Ebenfalls bei hohem pH-Wert wurde Al-Toxizität beschrieben, doch auch hier sind die Mechanismen noch nicht geklärt, genauso wenig wie die Al-Spezies, die die Toxizitätssymptome auslösen.

In der vorliegenden Arbeit wurde die Rolle von Si, B und pH-abhängiger Al-Spezifikation auf die Ausbildung von Al-Toxizitätssymptomen an der Al-sensitiven Maissorte ‚Lixis‘ untersucht. Dabei wurden folgenden Ergebnisse erzielt:

(1) Si Gaben während der Al-Behandlung, nicht aber eine Vorbehandlung mit Si führte zu einer Reduktion der Al induzierten Schädigung der Wurzel, was sich durch eine geringere Wurzelwachstumshemmung und Callosebildung bemerkbar machte. Da das Si-Angebot die Konzentration an monomerem Al nicht beeinflusste, kann man von einem *in-planta* Effekt von Si auf die Al-Resistenz ausgehen. Eine fraktionierte Analyse von Si und Al im ersten cm der Wurzelspitze zeigte, das 85% des Aluminiums in der Wurzelspitze in der Zellwand gebunden ist. Al Gehalte in der Apoplastenflüssigkeit, dem Zellsaft und der Zellwand unterschieden sich nicht zwischen +Si und –Si behandelten Pflanzen. Aber eine Al Behandlung erhöhte die Akkumulation von Si in der Zellwand und verringerte so die Mobilität von Al im Apoplasten. Diese

Ergebnisse deuten darauf hin, dass eine Behandlung mit Si zur Bildung von Hydroxylaluminiumsilikaten (HAS) im Apoplasten kommt und Al daher seine toxische Wirkung nicht mehr entfalten kann.

(2) Mit den hier untersuchten Parametern Wurzelwachstum und Callosebildung konnte kein Hinweis auf eine meliorierende Wirkung von B auf die toxische Wirkung von Al gefunden werden. Unterschiedliche B-Gaben beeinflussten auch nicht den Al Gehalt der Wurzelspitzen. Der B-Gehalt konnte nur durch sehr hohe B-Gaben erhöht werden und wurde durch die Al-Behandlung nicht beeinflusst. Die in der Literatur dokumentierten B/Al Interaktionen könnten auf eine Interaktion von B und Al im Pektinnetzwerk zurückzuführen sein. Man kann daher vermuten, dass auf Grund des geringen Anteils an Pektin in der Zellwand der Gräser der Gesamteffekt von B in Gräsern zu gering ist, um einen signifikanten Effekt auf die Al-Toxizität zu haben.

(3) Al reduzierte das Wurzellängenwachstum in gleichem Masse sowohl bei pH 4,3 als auch bei pH 8,0, obwohl die Konzentration an monomerem Al bei pH 8,0 um das vierfache niedriger war als bei pH 4,3. Nach einer 12 stündigen Al-Behandlung war der Al-Gehalt des ersten cm der Wurzelspitzen bei pH 8,0 im Vergleich zu pH 4,3 deutlich erhöht. Im Gegensatz dazu war die Al induzierte Callosebildung nur sehr gering und die Integrität des Wurzelgewebes war bei pH 8,0 besser erhalten. Unabhängig von den Versuchsbedingungen wurde der größte Teil des Aluminiums in der Zellwandfraktion gefunden. Ein signifikant geringerer Anteil des Aluminiums konnte in der sauren und basischen Waschlösung gefunden werden, ein höherer Anteil wurde im Zellsaft der unter alkalischen Bedingungen kultivierten Pflanzen gefunden. Eine sequentielle Extraktion der Zellwand mit steigenden Konzentrationen an KOH, lässt die Schlussfolgerung zu, bei diesen Kulturbedingungen der größte Anteil des Zellwandaluminiums als gefälltes $\text{Al}(\text{OH})_3$ vorliegt. Dies ist wahrscheinlich auf eine drastische pH Reduktion im Apoplasten bei einem pH der Nährlösung von 8,0 zurückzuführen. Diese Ergebnisse legen die Schlussfolgerung nahe, dass die Erhaltung eines sauren Apoplasten auch bei einem hohen pH-Wert der Nährlösung zur Bildung kationischer Al-Hydroxydspezies und $\text{Al}(\text{OH})_3$ führt, die zwar das Wurzelwachstum hemmen, aber zu einer geringeren Schädigung der

Plasmamembran als Al^{3+} , der bei niedrigem pH vorherrschenden Al Spezies, führen.

Die hier dargestellten Ergebnisse zeigen, dass Al in der Wurzelspitze hauptsächlich im Apoplasten vorkommt. Unterschiedliche Al-Spezies sind nicht in gleichem Masse toxisch. Das stark positiv geladene mobile Al^{3+} ist effizienter in der Induktion der Callosebildung, und der Reduktion der Zellintegrität und des Wurzelwachstums. Die Bildung von HAS und weniger stark positiv geladener Al-Hydroxydspezies reduziert die Al-Toxizität im Apoplasten.

Schlagwörter: Aluminiumtoxizität, Apoplast, Mais

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ABBREVIATIONS

Al	aluminium
Al _{mono}	monomeric aluminium
Al(OH) ₃	aluminium hydroxide
AWS	acid wash solution
B	boron
BaCl ₂	barium chloride
BWS	base wash solution
°C	degree Celsius
CaCl ₂	calcium chloride
CEC	cation exchange capacity
cm	centimetre
cv	cultivar
CW	cell wall
d	day
DTZ	distal transition zone
EWS	ethanol wash solution
Fig	figure
H ₃ BO ₃	boric acid
g	gram
GAX	glucuronoarabinoxylans
GFAAS	graphite furnace atomic absorption spectrometer
h	hour
H ⁺	proton
ha	hectare
HAS	hydroxyaluminiumsilicates
HCl	hydrochloric acid
HF	hydrofluoric acid
HNO ₃	nitric acid
HPLC	high performance liquid chromatography
H ₄ SiO ₄	silicic acid

ICP-OES	inductively coupled plasma optical emission spectroscopy
KCl	potassium chloride
KOH	potassium hydroxide
M	mol / litre
min	minute
mL	millilitre
mm	millimetre
mM	millimol / litre
n	number of observations
Na ₃ citrate	sodium citrate
NaOH	sodium hydroxide
nm	nanometre
nmol	nanomol
ns	statistical not significant
OH ⁻	hydroxide
p	probability
P	phosphorous
PE	pachyman equivalents
pH	negative logarithm of proton concentration
s	second
SD	standard deviation
Si	silicon
SS	symplastic sap
µg	microgram
µM	micromol / litre
v/v	volume / volume
WFSF	water free space fluid
%	percent

INTRODUCTION

Maize is an annual grass that ranks first in the world production of cereal crops (FAO, 2003). This plant species prefers soils with a pH between 5.5 and 8.0, however, the optimum pH should range from 5.5 to 7.0. If it is grown in soils with a pH below 5 and high Al supply, the yield becomes severely reduced (Lidon and Barreiro, 2002).

Composing 8% of the earth's crust, Al is the most abundant metal and the third most abundant element after oxygen and silicon (Martin, 1988). Aluminium is often found in combined form in soils and minerals as oxides and more commonly as complex Al silicates. Despite its abundance in the earth's crust, Al is generally not regarded as an essential element for plant growth. In contrast, the toxic and inhibitive effects of Al on the growth and development of plants are well known (see review Taylor, 1991; Horst, 1995; Kochian, 1995; Delhaize and Ryan, 1995; Taylor, 1995; Rengel, 1996; Matsumoto, 2000; Kochian et al., 2002).

Aluminium species and toxicity

Aluminium in the solid state plays a key role in our environment as a soil constituent. Furthermore, an understanding of its properties in solution is essential to formulate possible mechanisms for its interaction with cellular components, since cells of living tissues represent an electrolyte system (Haug, 1984). According to the chemistry of Al as highlighted by Martin (1988), in solutions more acid than pH 5, Al exists as the octahedral hexahydrate, $\text{Al}(\text{H}_2\text{O})_6^{3+}$, often abbreviated as Al^{3+} . As a solution becomes less acid, $\text{Al}(\text{H}_2\text{O})_6^{3+}$ undergoes successive deprotonation reactions yielding $\text{Al}(\text{OH})_2^+$ and $\text{Al}(\text{OH})_3$. Neutral solutions give an $\text{Al}(\text{OH})_3$ precipitate that redissolves in basic solutions due to the formation of the tetrahedral $\text{Al}(\text{OH})_4^-$. Polynuclear species may also form depending on the reaction time. Bioavailability of Al and toxicity for plant is associated with the pH of the solution surrounding the plant roots, since Al is soluble and biologically available in acid soils and waters, and biologically inactive at pH values around neutrality. In alkaline soils and

solutions, the solubility of Al increases, but its bioavailability is poorly known (Sparling and Lowe, 1996). It is now well understood that the toxicity of Al in aquatic and terrestrial system is not well correlated with total Al concentrations, but is a function of the concentration of the biologically active fraction in solution (Lewis, 1989). In terms of acute toxicity, the inorganic monomeric forms of Al are believed to be the most toxic. However, organically bound species may be capable of crossing biological membranes and contribute to chronic bioaccumulation of Al.

Effects of Al in the environment are highly dependent upon the form in which the element enters the system (Lewis, 1989). Although there is some uncertainty relating to the phytotoxicity of the various hydroxy-Al species (Kinraide, 1991; Taylor, 1995), it is believed that in acid soils and solutions Al^{3+} is the main Al species causing phytotoxicity (Kinraide et al., 1992; Matsumoto, 2000). But Al toxicity also exists in high pH soils amended with alkaline fly ash (Jones, 1961; Rees and Sidrak, 1955) and bauxite residue (Fuller and Richardson, 1986). In addition, it has been clearly demonstrated in high pH hydroponic culture media (Ma et al., 2003; Eleftherios et al., 1993; Kinraide, 1990; Fuller and Richardson, 1986). The Al species responsible for Al toxicity at high pH are not well known because of the complex micro-environment of root tip apoplast under such conditions.

The mechanisms of Al toxicity

Aluminium toxicity was implicated as early as 1918 as a cause of the inhibition of root growth of barley and rye in an acid soil (Hartwell and Pember, 1918). Despite intense research efforts on Al toxicity in the past decades, the full clarification of the mechanisms of Al toxicity has not been achieved. However, much progress has been made in understanding the effect of Al on the physiology and molecular biology of plants (see review Horst, 1995; Kochian, 1995; Delhaize and Ryan, 1995; Taylor, 1995; Rengel, 1996; Matsumoto, 2000; Rengel and Zhang, 2003).

Symptoms of aluminium toxicity

Aluminium primarily affects the plant roots (Horst, 1995). The most common Al toxicity symptoms are the inhibition of root elongation (Horst, 1987; Horst and Klotz, 1990, Horst et al., 1990; Zhang and Jessop, 1998), inhibition of lateral root formation (Hecht-Buchholz and Foy, 1981; Horst, 1987; Larsen et al., 1997; Blancaflor et al., 1998) and root hair development (Wood et al., 1984; Hecht-Buchholz et al., 1990; Brady et al., 1993; Care, 1995; Jones, et al., 1995; Jones, et al., 1998). The root system as a whole is coralloid in appearance, with many small stubby and brittle lateral roots but lacking fine branching (Foy et al., 1978; Furlani and Clark, 1981; Pavan and Bingham, 1982). The primary site of Al injury is the root apex. Particularly the first 5 mm root tip is the main site of Al accumulation and toxic effects (Ryan et al., 1993; Sivaguru and Horst, 1998).

Aluminum-caused damage of root tips might be explained by an inhibitory effect on cell division and cell elongation (Horst et al., 1983; Horst and Klotz, 1990). Al can inhibit root growth within 1 h (Kollmeier et al., 2000). Measurement of the inhibition of cell division by Al needs relatively longer Al treatment, because the cell cycle in roots is approximately 24 h (Powell et al., 1986). Thus, during the initial stages of Al inhibition of root growth, Al interactions with cell elongation must play a primary role (Kochian, 1995).

Aluminium alters root-cell ultrastructure depending on the type of tissues, on the developmental stage, and particularly on the position of the cells with respect to the Al source (Čiamporová, 2002). Almost regularly the cells at the root cap periphery, the cells of root epidermis and outer cortex undergo more drastic changes than the cells of inner cortex and central cylinder. Incipient symptoms of Al toxicity in the root tips of maize are an increase of vacuolar volume (Budíková, 1999; Čiamporová, 2002), destruction of root cap cells, swelling and destruction of epidermal and cortical cells resulting in a disintegrated outer shape (Hecht-bucht and Foy, 1981; Bennet et al., 1985; Budíková, 1999). Callose formation in the root tip is a sensitive marker for Al-induced injury (Horst et al., 1997). It has been demonstrated that Al-induced callose inhibits cell-to-cell trafficking of solutes through plasmodesmata (Sivaguru et al., 2000).

Primary sites of Al toxicity

The molecular mechanisms underlying Al toxicity are not yet well understood. Because Al forms strong bonds with oxygen-donor compounds, it can interact at multiple sites in the apoplast and symplast of root cells (Ma et al., 2001). The binding of Al with these substances is probably an important factor in its toxicity. However, the direct actions of Al on root cells are still unclear, particularly the relative importance of symplastic versus apoplastic lesions of Al toxicity remains a matter of debate. There is no consensus on the cellular site of Al toxicity, but many reports demonstrated that a major part of Al is located apoplastically (Clarkson, 1967; Horst et al., 1983; Marienfeld and Stelzer, 1993; Marienfeld, et al., 1995; Chang et al., 1999; Marienfeld et al., 2000; Taylor et al., 2000; Ishikawa et al., 2003). It has been shown in a giant alga that 99.99% of the total Al is located in the apoplast and only 0.01% in the symplast (Rengel and Reid, 1997). On the other hand, some researchers concluded from their studies that significant amount of Al is located in the symplast (Matsumoto et al., 1976; Tice et al., 1992; Lazof et al., 1994; Victor and Haug, 1996; Vázquez et al., 1999; Kataoka and Nakanishi, 2001). Even though agreement on the location of the majority of Al in the cell may be achieved, this would not entirely clarify the mechanism of Al toxicity, because it may be that the location of Al accumulation in the cell does not reflect the primary site of Al toxicity. Several questions remain to be answered: in which compartment is Al more harmful in causing cell growth-inhibition and cell death, in which form does Al exist in the apoplast and symplast, which Al form is phytotoxic in the apoplast and the symplast?

Cell wall

Aluminium strongly binds to the cell wall of root epidermal and cortical cells (Delhaize et al., 1993a). This is mainly due to the negative charge properties of the pectic matrix of cell walls (Blamey et al., 1990) which determine cation binding and distribution in the apoplast and thus at the outer surface of the plasma membrane (Kinraide et al., 1992; Horst et al., 1999). It has been shown that Al-resistant plants often have a lower root cation-exchange capacity (CEC) (Vose and Randall, 1962; Mugwira and Elgawhary, 1979; Blamey, et al., 1990; Kennedy, et al., 1986). However, some studies showed that the root cell-wall

CEC of the Al-resistant genotype was higher than that of the Al-sensitive genotype (Allan et al., 1990). Other studies also showed that CEC of the dry powder from the 1 cm root tip portion of the cultivars differing in Al resistance were similar in any of the plant species studied including rice, maize, pea, wheat and sorghum (Wagatsuma et al., 1997). So far, a common view has not been reached among researchers on whether root CEC plays a major role in Al sensitivity (see review Kochian, 1995).

The major differences in root CEC between monocots and dicots are not related to their respective Al resistance (Grauer and Horst, 1992), indicating that other factors such as release of Al-binding root exudates (Delhaize et al., 1993b; Basu et al., 1994; Pellet et al., 1995) are equally or even more important for genotypic differences in Al resistance (Horst et al., 1997). This was further confirmed by Wehr et al. (2003) who concluded that Al resistance conferred by low root CEC is not mediated by the ability to maintain pectin hydrolysis. Rather, exudation of organic acid anions can remove Al bound to pectin and this could alleviate toxicity, constituting a resistance mechanism.

Plasma membrane

Efforts to understand how plants respond to aluminium have focused on describing the symptoms of toxicity and elucidating mechanisms of resistance. However, little is known about the signal transduction steps that initiate the response of the plants (Sivaguru et al., 2003). Research has recently focused on early response of root tips to Al (Sivaguru et al., 1999; Nakanishi, 2001; Osawa and Matsumoto, 2001; Schmohl and Horst, 2002; Kataoka and Sivaguru et al., 2003; Ishikawa et al., 2003). The plasma membrane seems to play a major role in initial Al injury and resistance (see review Rengel and Zhang, 2003). Aluminium caused instantaneous plasma-membrane depolarisation in root cells of an Al-sensitive maize cultivar, and the intensity of depolarisation varied with the root-growth zones (Sivaguru et al., 1999). The rapid modification of the plasma membrane of the root-tip cells induced by Al affects the nutritional homeostasis of the cells (Ishikawa et al., 2003). Sivaguru et al. (2003) showed that Al depolymerises microtubules and depolarises the membrane. They proposed that signaling in response to Al is initiated by efflux of a glutamate-like

ligand through an anion channel and the binding of this ligand to a glutamate receptor.

Investigations on Al toxicity in plants have revealed that some plants detoxify Al in the rhizosphere by releasing organic acid anions (Miyasaka et al., 1991; Ryan et al., 1995; Yang et al., 2000; Kollmeier et al., 2001; Li et al., 2002; Mariano and Keltjens, 2003). In at least two species, wheat and maize, the transport of organic acid anions out of the root cells is mediated by aluminium-activated anion channels in the plasma membrane (Ryan et al., 1997; Ma et al., 2001; Kollmeier, et al., 2001).

Cytoskeleton

The growth inhibition and swelling of roots associated with Al exposure suggested that the cytoskeleton may be the target of Al toxicity (Blancaflor et al., 1998; Sivaguru et al., 1999). In the root elongation zone of maize, Al results in a reorganization of microtubules in the inner cortex, and an increase of the stability of the microtubules in the outer cortex cells coinciding with root-growth inhibition (Blancaflor et al., 1998). Sivaguru et al. (1999) demonstrated prominent Al-induced alterations in both the microtubular and the actin cytoskeleton especially in the apical 1-2 mm zone of an Al-sensitive maize cultivar. These alterations to the cytoskeleton were preceded by and/or coincided with Al-induced depolarization of the plasma membrane and with callose formation. Horst et al. (1999) suggested that the rapid disorganization of the cytoskeleton leading to root-growth inhibition is mediated by the interaction of Al with the apoplastic side of the cell wall - plasma membrane - cytoskeleton continuum.

Measures to ameliorate Al toxicity

In agricultural practice, many methods were developed to correct Al toxicity. Application of liming, fertilizer, and organic manure are generally essential for reduction of acidity-related constraints and to improve the crop production potential of acid soils (Baligar, et al., 1997). Each amendment has its own advantages. When more than two amendments are combined at a proper proportion, the beneficial effect of each amendment for crops could be enhanced (Baligar, et al., 1997).

Lime

The practice of liming, i.e., applying CaCO_3 , in order to raise the soil pH and precipitate exchangeable Al as insoluble, non-toxic $\text{Al}(\text{OH})_3$, has long been recognized as necessary for optimum crop production on acid soils (Haynes, 1984). However, in many acid soils large quantities of lime e.g. 2-10 t ha^{-1} , are commonly required to achieve adequate growth of many crops (Haynes and Mokolobate, 2001). Thus liming may not always be practical or cost-effective. Additionally, this amendment does not correct subsoil acidity.

Phosphorous

Phosphorous fertilizer supply can reduce Al toxicity and correct P deficiency commonly associated with acid soils. Because phosphate can complex soluble Al and bind protons, it may play an important role in Al resistance, both via complexation of Al^{3+} and by contributing to the alkalization of the rhizosphere pH, and hence decrease Al^{3+} activity (Pellet et al., 1997), in addition to raising concentrations of available soil P.

Organic residues

A number of reports demonstrate that additions of organic residues to acid soils can reduce Al toxicity and improve P availability (Berek et al., 1995; Slattery and Morison, 1995; Wong and Swift, 1995; Wong et al., 1995; Easterwood and Sartain, 1990; Hue et al., 1994; Iyamuremye et al., 1996; Haynes and Mokolobate, 2001). A wide range of organic compounds are released from the

residues and/or synthesized by the decomposer microflora during decomposition of organic residues. The two most important groups in relation to Al toxicity and P availability are soluble high molecular humic/fulvic acids and low molecular weight aliphatic organic acids. Both these groups of substances can complex and thus detoxify phytotoxic monomeric Al in the soil solution and they can also be adsorbed to Al and Fe oxides surfaces blocking P adsorption sites. Additionally, during crop residue decomposition, there is often a transitory increase in soil pH and this induces a decrease in exchangeable and soluble Al ions through their precipitation as insoluble hydroxyl-Al compounds (Haynes and Mokolobate, 2001).

Silicon

Silicon amelioration of Al toxicity in plants has been a research interest in recent years (Galvez et al., 1987; Li et al., 1989; Barceló et al., 1993; Baylis et al., 1994; Hammond et al., 1995; Ma et al., 1997; Cocker, 1997; Cocker et al., 1998a; Ryder et al., 2003). However the practice of supplement Si to ameliorate Al toxicity in soils has not yet been generally accepted because of the uncertainty of its effect. More specifically, the possible mechanisms of Si/Al interactions have not been conclusively examined.

Boron

Boron fertilizer application has been suggested as an alternative method to ameliorate Al toxicity in acid soils (Lenoble, et al., 1996a, 1996b). This may be more cost-effective than the existing amelioration methods. In addition, applied B readily penetrates into the subsoil, which is a great advantage because most of current amendments cannot rectify subsoil acidity. Thus the potential ability of B to reduce the toxic effects of Al on plants could be of interest and importance. However, the current information available to us about plant Al/B interaction is contradictory. There is no agreement on whether B ameliorates Al toxicity (Taylor and Macfie, 1994; Lenoble et al., 1996a, 1996b; Reid and Stangoulis, 2000).

The aim of this study was to develop a more convenient, cost-effective and environment-friendly methods to rectify Al toxicity. In addition, the information obtained could be helpful in understanding the complex mechanisms of Al toxicity, especially the role of the apoplast versus the symplast in Al toxicity. All the work was conducted with an Al-sensitive maize cultivar Lixis in solution culture. The first and second studies were carried out to investigate possible Al/Si (Chapter 1) and Al/B (Chapter 2) interactions. The last part dealt with Al toxicity at contrasting pH (Chapter 3) with different predominant Al species.

Chapter 1

Apoplastic binding of aluminium is involved in silicon-induced amelioration of aluminium toxicity in maize (*Zea mays* L.)

Abstract

The alleviating effect of silicon (Si) supply on aluminium (Al) toxicity was suggested to be based on ex or in-planta mechanisms. In my experiments with the Al-sensitive maize cultivar Lixis, Si treatment but not Si pretreatment ameliorated Al-induced root injury as revealed by less root-growth inhibition and callose formation. Si treatment did not affect monomeric Al concentrations in the nutrient solution suggesting an in-planta effect of Si on Al resistance. A fractionated analysis of Si and Al in the 1 cm root apices revealed that more than 85% of the root-tip Al was bound in the cell wall. Al contents in the apoplastic sap, the symplastic sap and the cell wall did not differ between -Si and +Si plants. Si did not affect the Al-induced exudation of organic acid anions and phenols from the root apices. However, Al treatment greatly enhanced Si accumulation in the cell wall fraction reducing the mobility of apoplastic Al. It was concluded that Si treatment leads to the formation of hydroxyaluminumsilicates (HAS) in the apoplast of the root apex thus detoxifying Al.

Keywords: Aluminium toxicity, apoplast, cell wall, silicon, maize

Introduction

Aluminium (Al) toxicity is one of the main factors limiting plant growth and crop yields in acid soils. Although much progress has been made during recent years, the mechanisms of Al-induced inhibition of root elongation and Al resistance are still not well understood. There are a number of excellent reviews in recent years summarising the state of knowledge and addressing knowledge gaps (Taylor, 1995; Kochian, 1995; Delhaize and Ryan, 1995; Matsumoto, 2000; Kochian et al., 2002). Particularly the relative importance of symplastic versus apoplastic lesions of Al toxicity remains a matter of debate. Rengel (1996) and especially Horst (1995) focussed the attention on the role of the apoplast in Al toxicity regarding short-term inhibition of root elongation by Al.

Silicon (Si) is a beneficial mineral element for plants and even a plant nutrient for some plant species (Epstein, 1999). The role of Si in plant resistance against biotic and abiotic stresses has been attributed particularly to modification of cell wall properties (Chérif et al., 1992; Fawe et al., 2001; Horst et al., 1999a; Lux et al., 2002). Iwasaki et al. (2002a, 2002b) and Rogalla and Römheld (2002) showed that Si-enhanced Mn leaf-tolerance is related to a reduction in the concentration of Mn^{2+} in the leaf apoplastic washing fluid in cowpea and cucumber, respectively. Si has been reported to alleviate Al toxicity in conifer (Ryder et al., 2003), barley (Hammond et al., 1995), soybean (Baylis et al., 1994), maize (Barceló et al., 1993), and sorghum (Galvez et al., 1987). Little or no effect of Si on Al resistance has been found in wheat, pea (Hodson and Evans, 1995) and cotton (Li et al., 1989). The beneficial role of Si has been suggested to be based on two aspects: solution chemistry and in-planta mechanisms (Cocker et al., 1998a). Ma et al. (1997) suggested that the ameliorative effect of Si on Al toxicity resulted from decreasing the toxic Al^{3+} concentration in solution by forming Al-Si complexes. On the other hand, some researchers observed in-planta effects of Si on Al resistance (Hammond et al., 1995; Corrales et al., 1997; Kidd, et al., 2001). Kidd et al. (2001) suggested that an enhanced exudation of phenolic compounds leading to complexation and thus detoxification of Al is responsible for the Si-mediated enhanced Al resistance in maize. More recently, Ryder et al. (2003) concluded from their work with *Picea abies* seedlings, that the amelioration of Al toxicity by silicon

could best be explained by a combination of both, bulk solution and in-plant effects.

The majority of the work on Si effects on plant Al resistance has been focused on the whole root and/or shoot system with relative long Al treatment periods, usually several days (Hodson and Evans, 1995). However, Al phytotoxicity expresses within minutes and hours in the root apices (Sivaguru and Horst, 1998). Therefore, the objective of this study was to better understand short-term effects of Al on root injury with special emphasis on Al/Si interactions in the root apoplast, which is the primary target of Al (Horst, 1995; Horst et al., 1999b).

Materials and methods

Plant material, growth conditions and experimental treatments

Seeds of an Al-sensitive maize cultivar Lixis were soaked in tap water overnight, then placed between filter-paper moistened with basic solution containing 500 μM CaCl_2 and 8 μM H_3BO_3 and kept in a vertical position for three days. Uniform seedlings were transferred to plastic pots containing the above-mentioned solution. Half the number of plants was supplemented with 1.4 mM H_4SiO_4 . Silicic acid was prepared by passing potassium silicate through a column filled with a cation exchange resin (Bio-Rad, AG 50W-X8, 100-200 mesh).

One day after transplanting, the pH of the nutrient solution was stepwise adjusted (using a pH-stat system) to pH 4.3 within 12 hours. Then plants from both Si treatments were exposed to 0 or 25 μM AlCl_3 for 1 h or 12 h without Si [--Si, +-Si] or with Si [++Si], and solution pH was maintained at 4.3 ± 0.1 thus avoiding Al precipitation. All experiments were conducted in a growth chamber under controlled environmental conditions of a 16/8h day/night cycle, 30/27°C day/night temperature, 75% relative air humidity and a photon flux density of 230 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active radiation at the plant height.

Root growth determination

For short-term root-elongation measurement, plant roots were stained in 0.5 % neutral red (pH 5.6) for 10 min before Al treatment. At harvest, the length of the unstained part of the root tip was measured as root elongation during the treatment. For long-term root-length measurement, all culture procedures were the same as described above except extending the Al treatment to 44 h, and the solution was renewed once during the Al treatment period. At harvest, the whole root system was scanned. The root length and the number of root tips were measured using the software WinRHIZO image analysis (WIN MAC, Regent Instruments Inc, Quebec, Canada).

Analysis of monomeric Al concentration in nutrient solution

After treatment, the culture solutions were filtered immediately through 0.025 μm nitrocellulose membranes. Monomeric Al (Al_{mono}) concentrations were measured colorimetrically using the aluminon method according to Kerven et al. (1989). The Al_{mono} concentration of the nominal 25 μM Al treatment solution was 20 μM after the 12 h Al treatment. There was no difference between the Si treatments (data not shown), suggesting that Si application did not lead to precipitation of Al in the treatment solution.

Root sample collection

After treatment, plant roots were rinsed with deionised water, 1 or 4 cm root tips (depending on the experiment) were excised using a razor blade. 1 cm root tips were frozen immediately in liquid nitrogen for callose determination. Individual 1 cm root segments were dissected from 4 cm root tips, and the root segments were stored at 4°C for Si and Al analysis.

Fractionation of Al and Si in root tips

The apoplastic and symplastic saps of the root tips were collected by centrifugation, according to the method described by Yu et al. (1999) with some modifications (Iwasaki et al., 2002c). Briefly, freshly excised 1 cm root tips from 20 seedlings were arranged in a filter unit (Millipore Ultrafree-MC, 0.45 μm) with

the cut ends facing down, the water free space fluid (WFSF) was collected by centrifugation at 3000 g at 4°C for 15 min. After collecting the WFSF, the root tips were frozen at -20°C. The symplastic¹ fraction was recovered from the frozen-thawed samples by centrifugation at 3000 g at 4°C for 15 min. The residue was transferred to Eppendorf vials and then the samples were homogenized in 1 mL ethanol with a mixer mill (MM200, Retsch, Haan, Germany) at a speed of 30/s for 3 min. After centrifugation, the supernatant and pellet were separated, the pellet was washed again with ethanol. The combined two supernatants represented the symplastic² fraction. The pellet consisted of the cell-wall material (CW).

Extraction of Al from cell wall

Aluminium was extracted from the cell wall on a Millipore filtration unit by a sequential procedure using solutions of 50 mM BaCl₂ (pH 4.3) for 5, 10 and 15 min, followed by 33 mM Na₃citrate (pH 5.8) for 5, 10 and 15 min. The Al contents in the BaCl₂ and Na₃citrate solution were determined by GFAAS (Unicam 939 QZ, Analytical Technology, Cambridge, UK).

Aluminium quantification

For Al analysis, the root segments or different fractions of root tips were wet digested with ultrapure concentrated HNO₃ at 135°C for 35 min in a microwave oven (MLS-ETHOS plus, Mikrowellen-Laborsystem, Leutkirch, Germany). After dilution with ultrapure water, Al concentrations in the solutions were quantified by ICP-OES (Spektro Analytical Instruments, Kleve, Germany) or GFAAS (Unicam 939 QZ, Analytical Technology, Cambridge, UK).

Callose quantification

Three 1 cm root tips were homogenized in 500 µL 1 M NaOH for 2 min at a speed of 20/s with a mixer mill (Retsch MM 200, Haan, Germany). After homogenization another 500 µL 1 M NaOH was added and callose was extracted for 30 min at 80°C in a water bath. Callose was quantified fluorometrically (Hitachi f2000, Hitachi, Tokyo, Japan; excitation 393 nm and

emission 484 nm) according to Köhle et al. (1985), using aniline blue as colour reagent. Pachyman (Calbiochem, Deisenhofen, Germany) was used as calibration standard. Hence, callose content was expressed as pachyman equivalents (PE) per root tip.

Silicon quantification

Silicon in the root segments or in different fractions of root tips was extracted by a mixture of 1 M HCl and 2.3 M HF (1:2 v/v). Si concentrations in the extract were determined colorimetrically (μ Quant Microplate Spectrophotometer, Bio-Tek Instruments, Winooski, Vermont, USA) according to the method described by Van der Vorm (1987).

Root exudates collection and determination

For the collection of organic acid anions and total phenols exuded from root apices, we employed the method described by Kollmeier et al. (2001). Briefly, roots of 10 intact 5-days-old seedlings were bundled. The tips (10 mm or 20 mm) were incubated for 2 h in 5 mL of a solution containing 500 μ M CaCl₂ and 8 μ M H₃BO₃ with different Al and Si levels according to the treatments. The rest of the roots was kept moist by wrapping them in filter paper soaked with basic solution. For the quantification of the exudation of organic acid anions the incubation was performed in filtration columns (Bakerbond SPE, J. T. Baber, Phillipsburg, USA) loaded with 1 g of an anion exchange resin (AG 1-X8, 100-200 mesh; Bio-Rad Laboratories, Hercules, USA). After removing the roots, the incubation medium was passed through the exchange resin at a rate of 1 mL min⁻¹. The organic acid anions adsorbed on the resin were eluted with 10 mL of 8 M formic acid. The formic acid was evaporated in a centrifugal evaporator (RCT 10-22T, Jouan, Saint-Herblain, France). The residue was dissolved in 1 mL perchloric acid (10 mM), and was then filtered through 0.45 μ m filtration units (Ultrafree-MC, Millipore, Eching, Germany). Samples were analyzed by isocratic HPLC (Kroma System 3000, Kontron Instruments, Munich, Germany) separated on an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, USA), supplemented with a cation micro-guard cartridge, using 10 mM perchloric acid as eluent at a flow rate of 0.5 ml min⁻¹ at 35°C. Total phenols in

the root exudates were determined after concentration in a centrifugal evaporator using Folin-ciocalteu reagent according to Swain and Hillis (1959).

Morin staining of Al in root tip

Aluminium in the root tissue was localized by staining with morin. After 1 h Al treatment, root tips from both Si treatments were excised and washed in a solution containing 500 μM CaCl_2 and 8 μM H_3BO_3 , pH 4.3. Free-hand sections from the 1-3 mm zone behind the root apex were stained with 25 μM morin (pH 5.6) for 30 min at room temperature. After washing in distilled water, the sections were observed under a fluorescence microscope (excitation filter 395-440 nm, barrier filter 470 nm). Images were taken by a digital camera (Sony, DSC-S85) and then exported to Adobe photoshop 5.0.

Results

Short-term experiments

Effect of Al and Si on root elongation and callose formation

Aluminium inhibited root elongation to about 50% within 12 h of Al treatment (Fig. 1A). Si supply during pretreatment and the Al treatment period significantly reduced the impact of Al on root elongation, whereas silicon supply only during the pretreatment did not. Al greatly stimulated callose formation in the root apices (Fig. 1B). Al-induced callose formation reflected the ameliorative effect of Si supply during pretreatment and Al treatment on root injury even more clearly. Again, Si supply only during pretreatment did not enhance plant Al resistance as revealed by the non-affected callose formation.

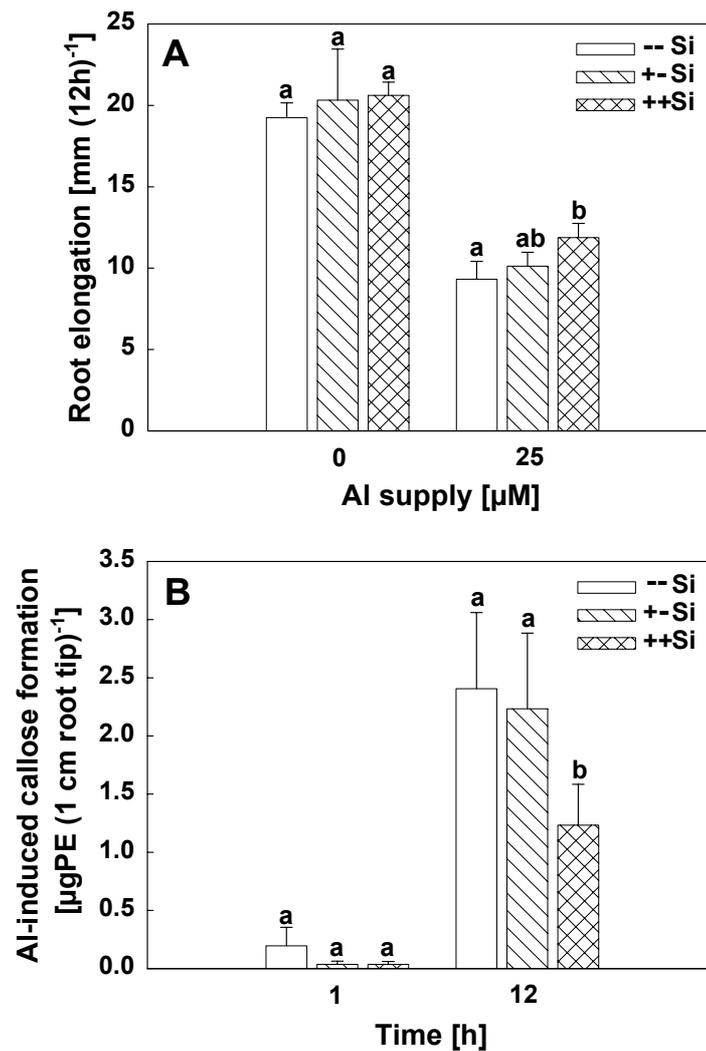


Figure 1. Effect of Si on root elongation (A) and on Al-induced callose formation (B) of maize cv Lixis supplied without or with 25 μM Al in a solution containing 500 μM CaCl_2 and 8 μM H_3BO_3 , pH 4.3. Plants were precultured for 36 h without or with 1.4 mM Si and then treated without or with 25 μM Al for 1 h or 12 h in the presence or absence of 1.4 mM Si. --Si: without Si during preculture and Al treatment, +-Si: with Si during preculture, without Si during Al treatment, ++Si: with Si during preculture and Al treatment. Bars show standard deviation. Significant differences between mean values are indicated by different letters at the $p < 0.05$ level (Tukey test). $n = 3$ for root elongation, $n = 5$ for callose formation.

Effect of Si on Al content in root segments

Aluminium contents in the root segments of the primary root tip were measured after 1 h and 12 h Al treatment (Fig. 2). Overall, there was no significant difference between Si treatments. Al contents in root segments increased with prolonged Al treatment. There was a significant difference between root segments. Root segments closer to the root apex accumulated higher amounts of Al.

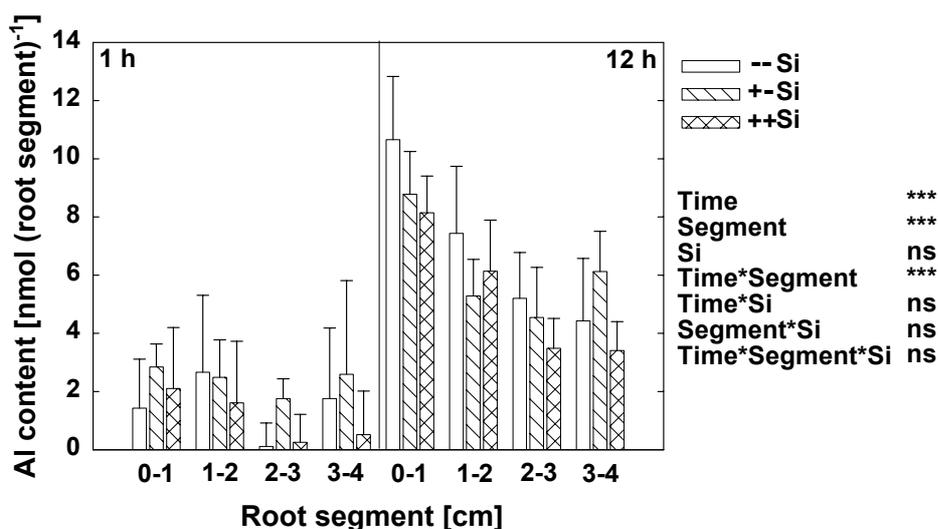


Figure 2. Aluminium contents of apical root segments of maize cv Lixis as affected by Si and Al supply grown in a solution containing 500 μM CaCl_2 and 8 μM H_3BO_3 , pH 4.3. Plants were precultured for 36 h without or with 1.4 mM Si and then treated without or with 25 μM Al for 1 h or 12 h in the presence or absence of 1.4 mM Si. Al content at 0 μM Al supply was subtracted from the 25 μM Al treatment. Bars show standard deviation, $n = 5$. *** indicates significance at the $p < 0.001$ level according to the F test. ns = non significant.

Effect of Al and Si on Si content in root segments

After 1 h and 12 h Al supply, Si contents in 1 cm root segments were measured (Fig. 3). The Si contents of the root tips of control plants (-Si treatment) were considered as background value. Si contents of the root segments of Si-treated plants gradually increased from the apical to the more basal root sections in all treatments (Fig. 3). After 1 h growth in Si-free solution, the Si contents of all root sections were significantly above the background level (Fig. 3A). However, after 12 h growth the Si contents of all root sections decreased, in the apical 1 cm even to the background level. This shows that Si accumulated during the pretreatment period could not be transferred apically to the newly formed root tips. In presence of Si also during the Al treatment period (Fig. 3B), the Si contents in all root segments were well above the background level. After 1 h Al treatment, Si contents of root segments were slightly higher in presence of Al (significant only for the root zones 1-2 and 2-3 cm). But after 12 h, the Si contents of all Al-treated root zones, particularly the root apex were clearly higher than those not treated with Al. Thus, it appears that the presence of elevated Si contents in the root apex is a prerequisite for the ameliorative effect of Si on Al toxicity.

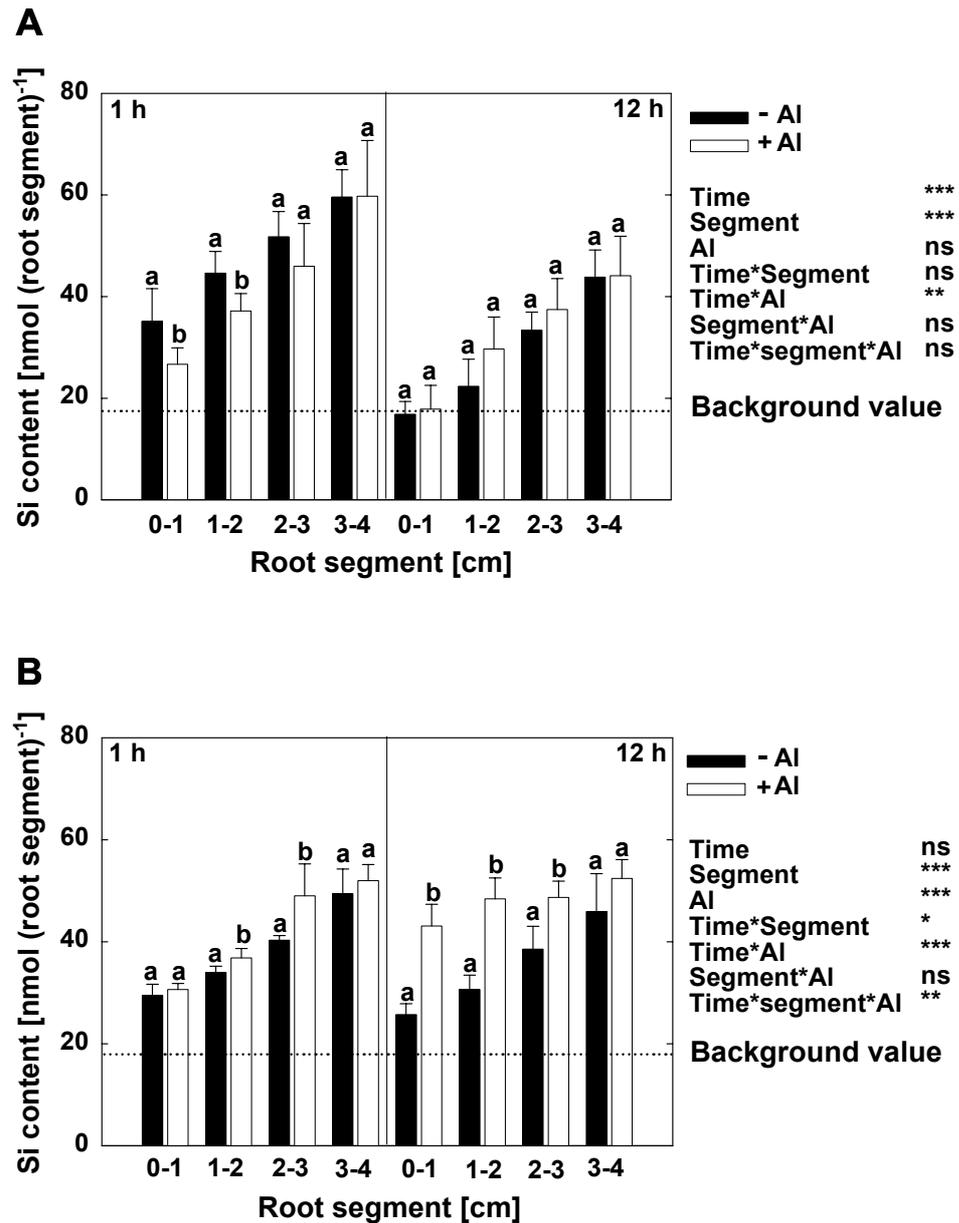


Figure 3. Silicon contents of root segments of maize cv Lixis as affected by Si and Al supply grown in a solution containing 500 μM CaCl_2 and 8 μM H_3BO_3 , pH 4.3. Plants were precultured for 36 h without or with 1.4 mM Si and then treated without or with 25 μM Al for 1 h or 12 h in the absence (A) or presence (B) of 1.4 mM Si. The background value (dashed line) presents the mean Si content of the root segments without Si treatment. Bars show standard deviation. Significant differences between mean values are indicated by different letters at the $p < 0.05$ level (Tukey test), $n = 5$. *, **, *** indicate significance at the $p < 0.05$, 0.01, and 0.001 level according to the F test. ns = non significant.

Fractionation of Al and Si in root tips

Since total root-tissue contents of Al and Si do not reveal their cellular distribution, their contents in different fractions of the apical 1-cm root tips were determined (Fig. 4). In Al-treated plants, only slightly higher Al contents could be found in the symplastic fraction. More than 85% of the root-tip Al was detected in the cell wall and thus the root apoplast (Fig. 4A). There was no significant difference between -Si and +Si plants in the Al content and its distribution. This indicates that the ameliorative effect of Si was not due to lower Al uptake into the root apex of maize.

Silicon treatment significantly enhanced Si contents in the symplastic fractions but not in the water free space fluid (WFSF) (Fig. 4B). Whereas the Si content of the cell walls was only slightly affected by Si supply in absence of Al, it was greatly increased in Al-treated plants. This is particularly well illustrated by the change of the relative distribution of Si between symplast and apoplast. In -Al plants, 81% of the total Si was localized in the symplast and only 19% in the apoplast, while in +Al plants, 53% of the total Si was in the apoplast and 47% in the symplast. This indicates that Si modifies Al binding to the cell-walls of root apices.

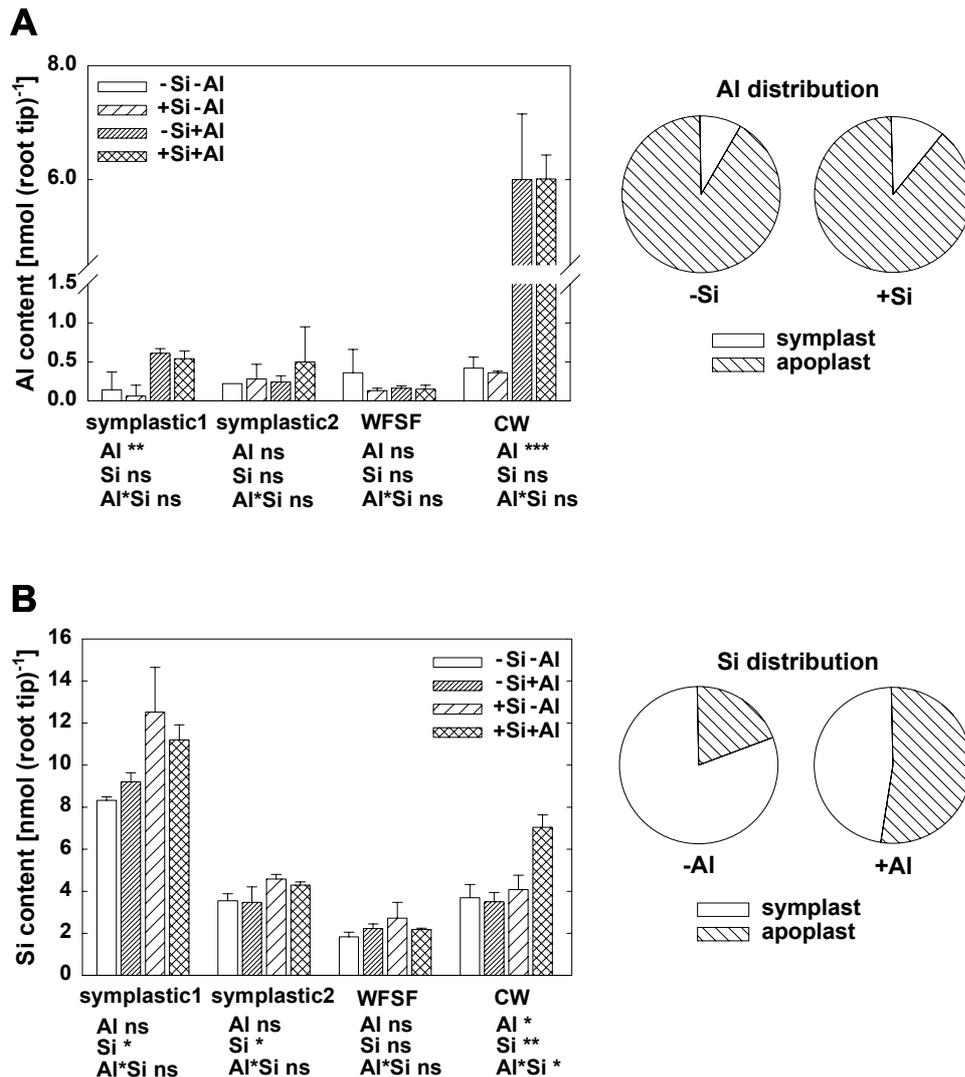


Figure 4. The contents (left) and relative distribution (right) of Al (A) and Si (B) in the symplast (symplastic1, 2), water free space fluid (WFSF) and cell walls (CW) of 1 cm root tips of maize cv Lixis as affected by Si and Al supply in a solution containing 500 μM CaCl_2 and 8 μM H_3BO_3 , pH 4.3. Plants were precultured for 36 h without or with 1.4 mM Si and then treated without or with 25 μM Al for 12 h in the absence or presence of 1.4 mM Si. Relative distribution after subtracting the background Al or Si contents in -Al or -Si treatments, respectively. Bars show standard deviation, $n = 3$. *, **, *** indicate significance at the $p < 0.05$, 0.01, and 0.001 level according to the F test. ns = non significant.

Effect of Si on the binding stage of Al in cell wall

The binding stage of Al in the cell walls of the root apex was studied using a fractionated desorption procedure with BaCl_2 followed by $\text{Na}_3\text{citrate}$ as extractants. With the exception of the first 5-min BaCl_2 -exchangeable Al fraction,

there were no differences between Si treatments (Fig. 5). The amount of readily BaCl₂-exchangeable cell-wall Al in -Si plants was higher than that in +Si plants.

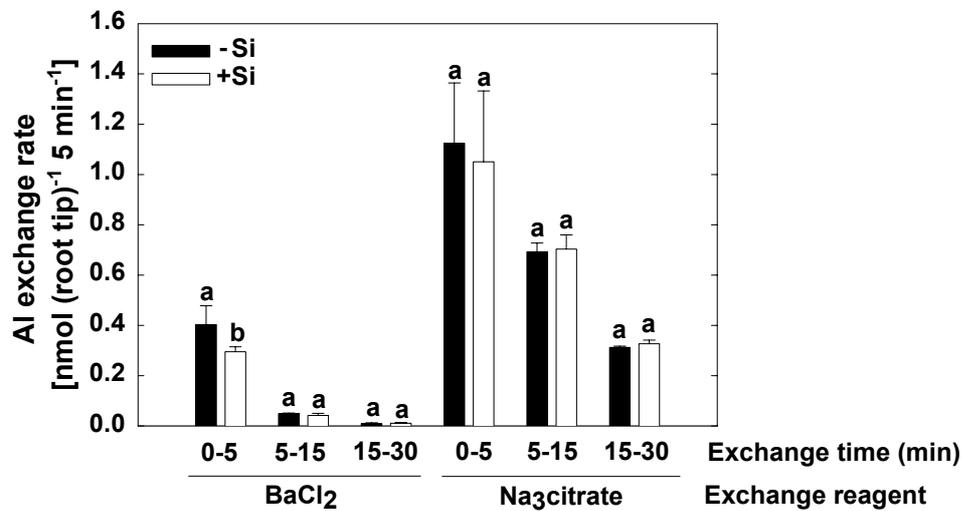


Figure 5. Aluminium exchange rate of cell walls isolated from 1 cm root tips of maize cv Lixis. Cell walls were desorbed sequentially in 50 mM BaCl₂ (pH 4.3) for 5, 10 and 15 min, followed by desorption in 33 mM Na₃citrate (pH 5.8) for 5, 10 and 15 min. Plants were precultured in a solution containing 500 μ M CaCl₂ and 8 μ M H₃BO₃, pH 4.3 for 36 h without or with 1.4 mM Si and then treated without or with 25 μ M Al for 12 h in the presence or absence of 1.4 mM Si. Bars show standard deviation, n = 4. Significant differences between mean values are indicated by different letters at the p < 0.05 level (Tukey test).

Morin staining of Al in root tip

Distribution and biological activity of Al was also studied using morin as a stain for Al in root cross-sections (Fig. 6). After 1 h Al treatment, Al entered up to three layers of the cortical cells. Bright fluorescence in the apoplast shows that the cell walls were the main sites of Al localization. Clear differences in Al distribution were visible between the Si treatments. Without Si supply, Al treatment resulted in a bright Morin-Al fluorescence of the outer tangential walls of all epidermal cells. In the +Si treatments, many epidermal cells were not fluorescent with the exception of some radial cell walls of epidermal cells.

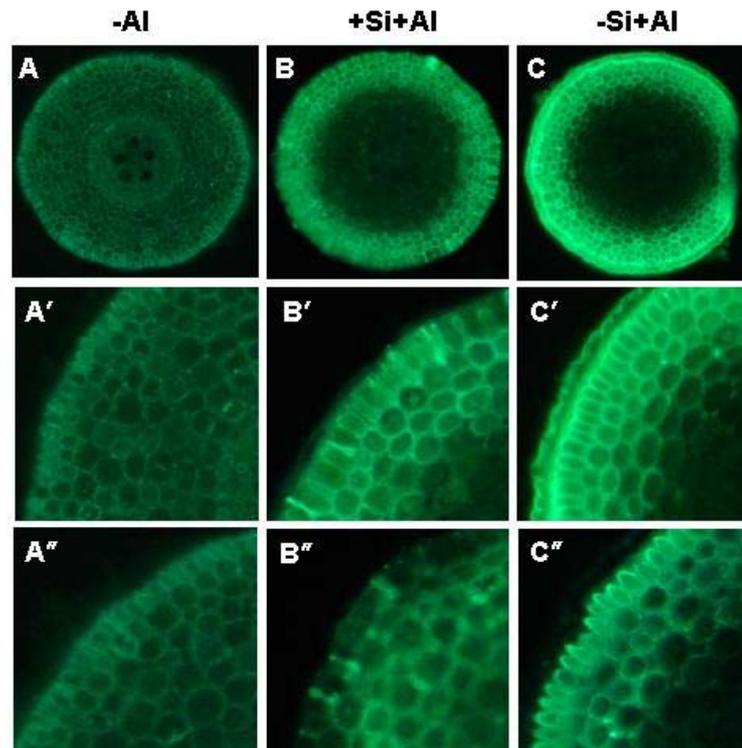


Figure 6. Fluorescence of the morin-Al complex in root cross sections of maize cv Lixis. Plants were precultured in a solution containing 500 μM CaCl_2 and 8 μM H_3BO_3 , pH 4.3 for 36 h without or with 1.4 mM Si and then treated without or with 25 μM Al for 1 h in the presence or absence of 1.4 mM Si. Excitation filter 395-440 nm, barrier filter 470 nm. A, B, C: Cross sections from the root zone 1-2 mm behind the root tip. A', B', C': Close-up of Al staining of epidermal and cortical cells in A, B, C, respectively. A'', B'', C'': Close-up of Al staining of epidermal and cortical cells in cross sections from the root zone 2-3 mm behind the root tip.

Effect of Al and Si on the exudation of organic acid anions

Organic acid anion exudation is a well-documented Al resistance mechanism in maize. In order to ascertain whether either Si, or Al and Si together interfere with this resistance mechanism, we determined the release of organic acid anions from root apices during short-term (2 h) Al treatment (Fig. 7). Al induced citrate exudation, but Si did not show a significant effect on citrate excretion of the root tips. There was even a trend of lower citrate release in Al and Si-treated plants, which may reflect less Al stress in presence of Si. Malate exudation was not affected either by Al or Si. No oxalate exudation was detected in this experiment.

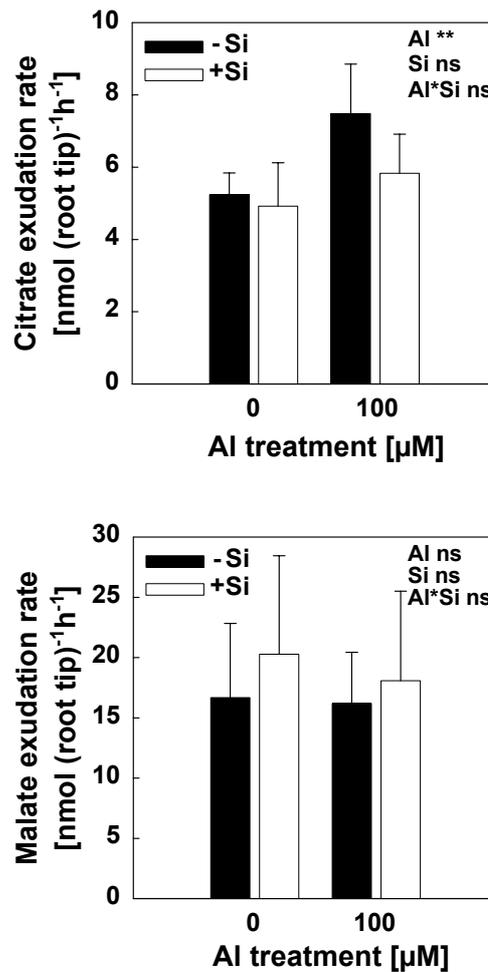


Figure 7. Citrate and malate exudation of 1 cm root tips of intact plants of maize cv Lixis. Plants were precultured in 500 μM CaCl_2 and 8 μM H_3BO_3 solution at pH 4.3 for 24 h without or with 1.4 mM Si. Then roots of 10 plants were bundled and the tips incubated for 2 h in 5 mL of the treatment solution with different Al and Si levels according to the treatments. Bars show standard deviation. Results of two experiments were combined and data are means of 6 replicates. ** indicates significance at the $p < 0.01$ level according to the F test. ns = non significant.

Effect of Al and Si on total phenol exudation

Because phenol exudation was reported to confer Si-induced Al resistance in maize (Kidd et al., 2001), we also investigated the effect of Si and Al on phenol exudation from root apices in my short-term experiments. The result showed that neither Al nor Si induced the phenol exudation (Fig. 8).

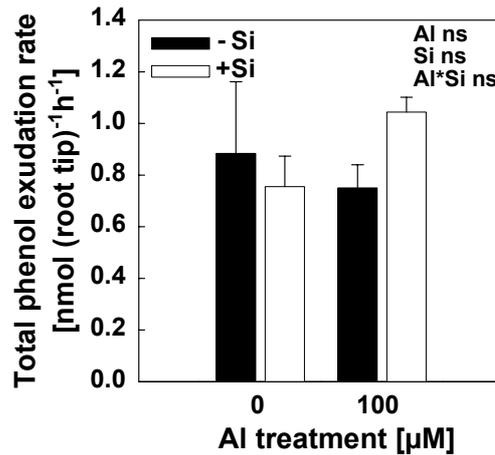


Figure 8. Total phenol exudation of 2 cm root tips of intact plants of maize cv Lixis. Plants were precultured in 500 μM CaCl_2 and 8 μM H_3BO_3 solution at pH 4.3 for 24 h without or with 1.4 mM Si. Then the roots of 10 plants were bundled and the tips were incubated for 2 h in 5 mL of the treatment solution with different Al and Si levels according to the treatments. Bars show standard deviation, $n = 4$. ns = non significant according to the F test.

Long-term experiments

Effect of Al and Si on root growth

The ameliorative effect of Si on Al toxicity was even clearer in long-term experiments (44 h Al treatment). Aluminium supply significantly decreased the root growth either in the presence or absence of Si as shown in Fig. 9A. Without Al supply, there was no difference between Si treatments. For the 25 μM Al treatment, total root length of +Si plants was higher than of -Si plants, which was mainly due to higher lateral root length of +Si plants (Fig. 9B). The number of root tips was decreased by Al supply (Fig. 9C). Si alone had no effect on the number of root tips, but Si enhanced the number of root tips under conditions of Al toxicity.

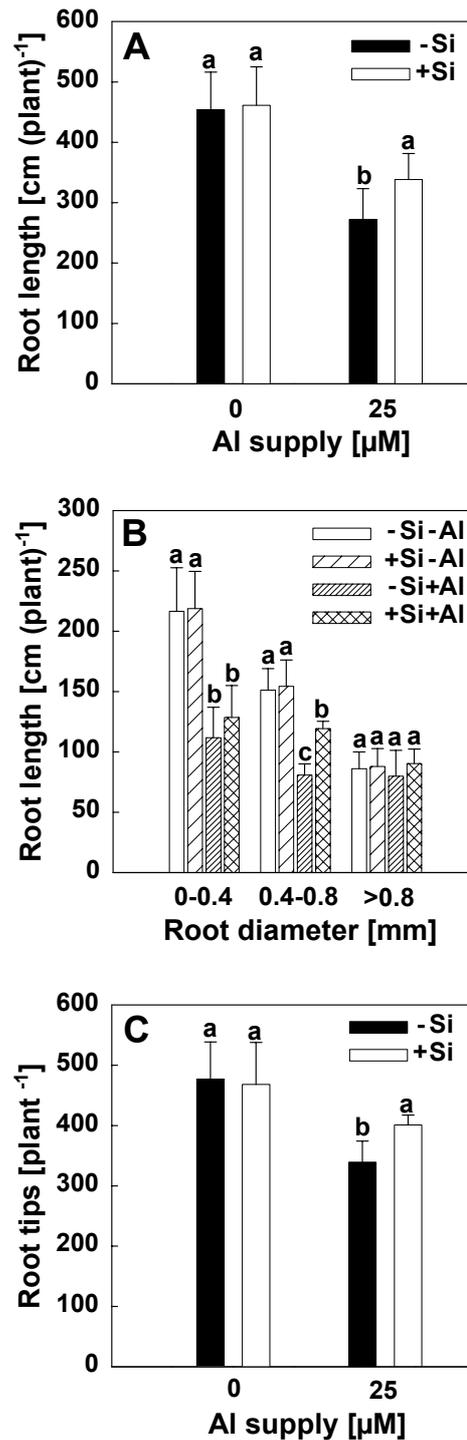


Figure 9. Effect of Si on total root length (A), root length of different root classes (B), and the number of root tips (C) of maize cv Lixis exposed to 0 and 25 μM Al in a solution containing 500 μM CaCl_2 and 8 μM H_3BO_3 at pH 4.3. Plants were precultured for 36 h without or with 1.4 mM Si and then treated without or with 25 μM Al for 44 h in the presence or absence of 1.4 mM Si. Bars show standard deviation, $n = 6$. Significant differences between mean values are indicated by different letters at the $P < 0.05$ level (Tukey test).

Discussion

The ameliorative effect of Si on Al toxicity in plants was attributed to a decreased availability of phytotoxic Al in the culture media by some authors (Baylis et al., 1994; Ma et al., 1997). This decrease in Al concentration is supposed to be due to the formation of biologically inactive complexes of hydroxyaluminumsilicates (HAS). Besides the chemical reaction in solution, in-planta detoxification was suggested based on experiments where amelioration was observed but HAS formation was minimal (Corrales et al., 1997; Kidd et al., 2001).

Solution chemistry of Al/Si interactions

A major problem in investigating Al and Si interactions in hydroponic culture over the last 15 years has been uncertainties concerning the chemistry of Al and Si in the solution in which the plants were grown (Ryder et al., 2003). It is well known that at neutral and moderately acid solution pH, Al and Si will form HAS, but Al toxicity mainly occurs at pH values below 5. Doucet et al. (2001) found that HAS were not identified in any solution in which the precipitation of $\text{Al}(\text{OH})_3$ was not predicted. A review by Exley et al. (2002) demonstrated that the formation of an aluminium hydroxide template was a prerequisite for HAS formation. These findings suggest that in acid solutions (pH 4.3) HAS formation is not a major factor, because only low concentrations of aluminium hydroxide exist in low pH solutions. Under my experimental conditions it can be assumed that the HAS formation in the Al treatment solution was low. The assumption is supported by the fact that I could not detect any changes in the concentration of inorganic monomeric Al, which has been shown to be the most physiologically active phytotoxic form of Al (Kerven et al., 1989). Therefore, I consider an in-planta effect as main contributing factor to the amelioration of Al toxicity by Si, under my experimental conditions.

Effect of Si on modifying apoplastic binding of Al

Horst (1995) proposed that the apoplast of the root tip is the primary site of Al phytotoxicity. Al binds rapidly to the negatively charged binding sites in the cell wall altering cell-wall properties and thus affecting root growth. Different mechanisms were discussed, how Si could exert its positive effect on Al resistance. Corrales et al. (1997) suggested that esterification of cell-wall components by Si reduces the binding of Al to the cell wall. Kidd et al. (2001) suggested that an enhanced exudation of phenolic compounds is responsible for the Si-induced Al resistance in maize. Both mechanisms would lead to reduced Al concentrations in the apoplast. Cocker et al. (1998b) proposed the formation of HAS in the apoplast, so that Al would be transferred into a non-phytotoxic form, without reducing the Al content. This conclusion is supported by the results of Hodson and Sangster (1993). Using X-ray microanalysis, they showed the co-presence of Si and Al in epidermal cells of sorghum roots treated with both Al and Si. In roots of Al and Si-treated wheat, Cocker et al. (1997) found Al and Si co-localized in epidermal and hypodermal cells.

In the experiments presented here, the total amount of Al in the cell wall, as well as in any other cell fraction was not changed by Si treatment (Fig. 4A), but the exchangeability of the cell wall-bound Al changed. The easily exchangeable Al fraction was reduced by Si (Fig. 5). Concomitant with this modification of Al binding I found a change in the cellular distribution of Si (Fig. 4B). Al treatment shifted the cellular Si distribution from the cytoplasm to the cell-wall fraction. These findings support the hypothesis that the formation of Al-Si complexes is responsible for the ameliorative effect of Si and are in agreement with the observation of Cocker (1997). In his study with wheat, the fluorescent dye morin was employed to localize Al. He showed that roots treated with both Al and Si were less fluorescent than roots treated with Al alone. Morin is believed to bind only to biologically active Al (Browne et al., 1990). Therefore his results suggested that although Si did not reduce the concentration of total Al, it might have reduced the concentration of biologically active Al within the cell wall. The morin staining method was also applied in the present study (Fig. 6). Instead of staining whole root tips after long-time Al treatment (Cocker, 1997) cross sections from the root apical 1 to 3 mm behind the root tip were examined after

1 h Al treatment. The results were generally in accordance with the results of Cocker (1997). After 1 h Al treatment, Al entered up to three layers of cortical cells and the most fluorescent compartment was the cell wall. In the presence of Si, the general staining of Al with morin was less intense with the exception of a few radial walls of epidermis. A possible explanation for this phenomenon could be that silicic acid on the root surface retarded Al accumulating on the root surface. The other possible explanation is the formation of HAS. High Al concentrations in the epidermis (Marienfeld et al., 2000) and the relative high pH (compared with the bulk solution) on the root surface of the DTZ (Kollmeier et al., 2000) will favour HAS formation, thus reduce the biologically active Al concentration. These results are consistent with the results of Cocker et al. (1997), who detected Al and Si co-deposits in the outer tangential walls of the root epidermis of wheat.

The morin technique cannot provide quantitative information on Al localization and binding stage in plant. Therefore, I applied a fractionation technique. In Si-treated roots the most mobile cell-wall Al fraction that could be desorbed with BaCl_2 within 5 min was significantly reduced by Si treatment. However, this fraction represented only a 2% difference between the Si treatments when related to the total Al content of the root tip. So the question arises, whether these 2% less loosely bound Al in the cell wall of Si-treated plants can account for the Si-amelioration effect observed? Obviously, this fraction and the WFSF fraction are characterized by a particularly high mobility in the apoplast. Therefore, it can be expected that part of these fractions were recovered in the symplastic¹ fraction during the extraction/centrifugation steps which then was overestimated at the expense of the apoplastic fractions. The mobile apoplastic fractions are expected to determine the Al activity at the plasma membrane and thus Al toxicity (Kinraide, 1994) as revealed by enhanced callose formation in the presence of Al (Fig. 1B).

Hodson and Wilkins (1991) localized Al in the roots of Norway spruce using X-ray microanalysis. They found that silicon concentrations in the cortical cell walls of the Al-resistant plants increased in response to Al treatment. Using an Al-sensitive maize cultivar, I found a similar response: the Si content of the apical 1 cm primary root increased with Al treatment. The results of the present study support the proposition, that Si exerts its beneficial effect on the

expression of Al-toxicity through the formation of non-phytotoxic HAS in the apoplast. This assumption is based on the fact that Si treatment leads to similar Al contents but less loosely bound Al in the cell walls of Al-treated root tips. Additionally, the ameliorative effect of Si on Al toxicity occurred only when sufficient Si was present in the root tips. When Si was applied to the plants only during pretreatment and the Si content of the 1 cm root apex was diluted by growth to the background Si level (Fig. 3A) no ameliorative effect of Si was found (Fig. 1). This finding conflicts with the results of Corrales et al. (1997). They showed that an Al-sensitive maize variety pretreated with Si and then exposed to Al for 24 h in the absence of Si showed higher root growth rates than plants not pretreated with Si, and the ameliorative effect of Si was due to lower Al uptake of the whole root system or mature root zones (approximately 5 cm from the root tip). The difference between these results may come from the different root zones that have been investigated in the two studies. I believe using root tips as the target for Al and Si interaction in plants is preferable, because the primary site of aluminium toxicity in maize is the root apex. Ryan et al. (1993) have shown that in maize, root elongation is inhibited only when apices are exposed to Al, whereas exposing the remainder of the root does not inhibit elongation. In the present study, I used the same maize cultivar Lixis, which has been intensively investigated by Sivaguru and Horst (1998), who showed that the DTZ (1 to 2 mm behind the root apex) is the primary target of Al.

Effect of Al and Si on root exudation

Kidd et al. (2001) observed the effect of Si pretreatment on Al resistance in maize. Under their experimental conditions phenol exudation was a major factor contributing to Si-enhanced Al resistance. Al and Si triggered the release of catechol and of the flavonoid-type phenols catechin, and quercetin. In an Al-resistant variety, Si-pretreated plants exuded more phenols than plants not pretreated with Si. In my short-term experiments, neither Al nor Si induced phenol exudation. It cannot be ruled out that in my experiments Al treatment time (2 h) was too short for the effects to occur. But considering that in Si-pretreated root tips, elevated Si contents could not be measured after the roots

had been growing in a Si-free solution for only 12 h, the enhanced phenol exudation reported by Kidd et al (2001) after 24 h growth in Si-free solution might be due to the release of phenols from more mature root zones. Also a genotype-specific response cannot be excluded.

With regards to organic acid anions, Al stimulated root exudation of citrate within two hours (Fig. 7), but the exudation was not affected by Si. This result is consistent with Cocker et al. (1998b). In their experiments to assess exudation of malate by roots of the wheat cultivar Atlas 66 treated with 100 μ M Al, the presence of Si was found to have a negligible effect on exudation after 24 h Al treatment. In the study of Kidd et al. (2001), Al stimulated root exudation of oxalic acid greatly in three maize varieties after 24 h Al treatment, this exudation was also not affected by Si pretreatment. It appears that organic acid anions do not play a significant role in Si-mediated amelioration of Al toxicity.

Conditions for HAS formation in root apoplast

Significant advances have been made in understanding the complex chemistry of Al and Si interactions in solution. However, little is known of Al reactions in the root apoplast, and the interactions of Al and Si in this compartment are likely to be even more complex (Cocker et al., 1998a). The formation of an Al-Si complex depends on pH, Al and Si concentration. In the nutrient solution I used, low pH and low Al concentration were not favourable for Al-Si complex formation (see above). But within the apoplast of the root apex, higher pH combined with high Al and Si concentrations (considering the small volume of the apoplast in the root tip) could promote HAS formation. Kollmeier et al. (2000) showed that the root surface pH of the root apex of maize cv Lixis grown in bulk solution with pH 4.5 was as high as 5.3 without Al supply. With Al supply the surface pH of the same root zone was decreased to 4.9 and 4.7 after 15 and 60 min respectively. Peters and Felle (1999) also observed that the root surface of the maize root apex was more alkaline than the solution at pH 4.2. It may be assumed that under my experimental conditions the apoplastic pH in the root tip was also higher than that on the root surface, because I observed an increase of the solution pH when the pH was not kept constant using a pH-stat during the experiment (data not shown). Cocker et al. (1998a) suggested that

the concentrations of Al and Si and pH within the apoplast are likely to decide HAS formation. The fact that no ameliorative effect of Si on Al toxicity was observed after 1 h Al treatment according to Al-induced callose formation, but a clear effect could be detected after 12 h Al treatment may reflect such dose requirement in cortical cell walls. In addition, the beneficial effect of Si on Al resistance was more pronounced in the long-term experiment (Fig. 9).

In conclusion, the ameliorative effect of Si on Al toxicity described here can be attributed to an in-planta effect. This effect is most likely due to the formation of HAS in the apoplast, which transforms Al into a non-phytotoxic form in the apoplast of the root apex.

Chapter 2

Assessing the effect of boron on aluminium resistance in maize (*Zea mays* L.)

Abstract

The effect of boron on Al resistance in an Al-sensitive maize cultivar Lixis has been assessed in hydroponic culture. Based on the performance of root growth and callose formation, no evidence was found for an ameliorative effect of B on Al toxicity. Various B supplies also did not affect the Al content in the root tip. B content in the root tip was only increased at very high B supply and was not influenced by Al treatment. The documented B/Al interaction might be due to the interaction of B and Al in the pectin network. From my results I concluded that due to the low pectin content of grasses the overall effect of B is too weak to have any significant influence on Al toxicity in grasses.

Keywords: Aluminium resistance, boron, maize

Introduction

Aluminium toxicity is one of the main factors limiting plant growth in acid soils (Foy et al., 1978). Many methods have been proposed to reduce Al toxicity, using Al-resistant cultivars, applying lime, phosphorus fertilizers or organic residues (Haynes and Mokolobate, 2001), but the more convenient and economic ways are still waiting to be explored.

One of the first symptoms of Al toxicity is an inhibition of root elongation. The distal part of the transition zone (DTZ, 1-2 mm from the root tip) is the most Al-sensitive apical root zone in maize (Sivaguru and Horst, 1998). Boron also primarily inhibits root elongation through limiting cell enlargement but not cell division (Brown et al., 2002).

Boron and aluminium interactions in plants have been proposed by several researchers, but most of these proposals were based on indirect evidence. Blevins (1987) proposed that Al could inhibit root growth by inducing B deficiency. Later, Lukaszewski and Blevins (1996) noted that both Al toxicity and B deficiency in cucurbit caused a reduction in ascorbate concentration in root apices that was correlated with reduced root growth. They proposed that Al toxicity was due to the impairment of the role of B in ascorbate metabolism. Poschenrieder et al. (1995) found that there was a significant correlation between the Al-induced increase in callose formation in root tips and the Al-induced inhibition of B uptake in a number of maize cultivars. A disturbance of the basipetal auxin flow has been shown for Al (Kollmeier et al., 2000) and an interference with IAA transport is also discussed for B (Marschner, 1995).

Based on the similarities of the symptoms characteristic of Al-stressed and B-deficient plants, it was proposed that Al may exert its toxic effect by inducing boron deficiency (Blevins, 1987; Blevins and Lukaszewski, 1998) and additional B supply may ameliorate Al toxicity (Lenoble et al., 1996a, 1996b).

If this assumption could be verified, B would be a viable candidate for the amelioration of Al toxicity in acid soils, since B supplementation is less costly than current methods of soil-acidity amelioration (e.g. liming). Moreover, B readily penetrates into subsoil zones (Lenoble et al., 1996a, 1996b), which could correct subsoil acidity limiting rooting depth and thus increase drought tolerance and the soil volume available for nutrient uptake.

Up to now, the assumption has been investigated only in a few cases. Taylor and Macfie (1994) conducted extensive experiments with an Al-sensitive variety of wheat in solution culture and found no evidence that B was capable of ameliorating Al toxicity. More recently, results from Reid and Stangoulis (2000) also showed no evidence that supplemental B ameliorated Al toxicity in wheat. However, Lenoble et al. (1996a, 1996b) found supplemental B could prevent Al-induced inhibition of root growth of squash in solution culture and of alfalfa in soil culture. It has been suggested that the positive effect of B on Al toxicity observed on squash and alfalfa and no effect on wheat may reflect the differences between dicot plants and grasses in their internal B requirement, or the different effects of Al on B nutrition between the species (Taylor and Macfie, 1994).

The objective of this study was to investigate the interaction between B and Al with respect to Al resistance in maize. Root elongation and callose formation was used to assess ameliorative effects of B on Al toxicity after short-term treatments. These parameters are well established, to allow a very sensitive assessment of the degree of Al stress experienced by plants, and should, therefore, make it possible to resolve some of the questions arising from the conflicting results on B/Al interaction.

Materials and methods

Plant material and growth conditions

Seeds of an Al-sensitive maize cultivar Lixis were sown on filter-paper rolls moistened with tap water. After four days, the uniform seedlings were transplanted to plastic pots containing 8 L 500 μM CaCl_2 solution with different B supply according to the treatments. After one day, the pH of the solution was stepwise adjusted to pH 4.3 within 24 h before Al treatment. All experiments were conducted in a growth chamber under controlled environmental conditions of a 16/8 h day/night cycle, 30/27°C day/night temperature, 75% relative air humidity and a photon flux density of 230 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photosynthetic active radiation at the plant height.

Root elongation measurement

Before treatment, plant roots were stained with 0.5% neutral red (pH 5.6) for 10 min. At harvest, root elongation was estimated by measuring the length of the unstained part of the root tip from the primary root and the longest secondary root.

Root sample collection

After treatment, plant roots were rinsed with deionised water, 1 cm root tips were excised using a razor blade, stored at 4°C for Al or B analysis or frozen immediately in liquid nitrogen for callose determination.

Callose quantification

Three 1 cm root tips were homogenized in 500 µL 1 M NaOH for 2 min at a speed of 20/s with a mixer mill (Retsch MM 200, Haan, Germany). After homogenization another 500 µL 1 M NaOH was added and callose was extracted for 30 min at 80°C in a water bath. Callose was quantified fluorometrically (Hitachi f2000, Hitachi, Tokyo, Japan; excitation 393 nm and emission 484 nm) according to Köhle et al. (1985), using aniline blue as colour reagent. Pachyman (Calbiochem, Deisenhofen, Germany) was used as calibration standard. Hence, callose content was expressed as pachyman equivalents (PE) per root tip.

Aluminium quantification

Root tips were wet digested with ultrapure concentrated HNO₃ at 135°C for 35 min in a microwave oven (MLS-ETHOS plus, Mikrowellen-Laborsystem, Leutkirch, Germany). After dilution with ultrapure water, Al concentrations in the solutions were quantified by ICP-OES (Spektro Analytical Instruments, Kleve, Germany).

Boron determination

Root tips were wet digested with ultrapure concentrated HNO₃ at 90°C for 6 h in a microwave oven (MLS-ETHOS plus, Mikrowellen-Laborsystem, Leutkirch, Germany). After dilution with ultrapure water, B concentrations in the solutions were quantified by the curcumin method according to Wimmer and Goldbach (1999).

Results

Influence of B and Al on root growth

The first experiment was conducted to determine the optimum B concentration range for proper root growth in the absence of Al (Table 1). Root elongation during 20 h was similar between 2 and 128 µM B. Without B supply, root growth was retarded, which indicated that B deficiency occurred. Primary roots and secondary roots did not show any significant differences in elongation rate at different B concentrations. Since B supply as high as 128 µM did not show B toxicity and B supply as low as 2 µM did not show B deficiency during the treatment, this B concentration range was used to investigate the Al/B interactions in maize.

Compared to non Al-treated plants (Table 1), aluminium treatment significantly reduced the root elongation in all B treatments (Table 2). Except for the zero B treatment, root elongation was similar for all B treatments. Comparing the root elongation during 11 h and 24 h Al treatment period, the increase in root length was 1.6-2.1 cm and 0.4-0.8 cm for the first 11 h and the next 13 h, respectively. Thus the reduction of root elongation by Al was progressively intensified with Al treatment duration.

Table 1. Influence of boron on root growth of maize cv Lixis. Plants were grown in 500 μM CaCl_2 solution with different B supply for 52 h. Root elongation was determined from 32 to 52 h. Data are means of three replicates, each replicate consisted of 12 plants. Values followed by same letters down the column are not significant different at the $p < 0.05$ level (Tukey test).

B supply [μM]	Root elongation [$\text{mm} (\text{h})^{-1}$]	
	Primary root	Secondary root
0	1.8 \pm 0.2 b	2.0 \pm 0.1 b
2	3.2 \pm 0.1 a	3.1 \pm 0.1 a
32	3.3 \pm 0.0 a	3.1 \pm 0.0 a
64	3.2 \pm 0.1 a	3.0 \pm 0.1 a
128	3.0 \pm 0.1 a	2.9 \pm 0.2 a

Table 2. Influence of Al on root elongation of maize cv Lixis as affected by different B supply. Plants were grown in 500 μM CaCl_2 solution with various B supply for 48 h and were then treated with 25 μM Al for 11 and 24 h in the presence of different concentrations of B. Data are means of three replicates, each replicate consisted of 12 plants. Values followed by same letters down the column are not significant different at the $p < 0.05$ level (Tukey test).

B supply [μM]	Root elongation (11h) [$\text{mm} (\text{h})^{-1}$]		Root elongation (24h) [$\text{mm} (\text{h})^{-1}$]	
	Primary root	Secondary root	Primary root	Secondary root
	0	1.4 \pm 0.1 b	1.5 \pm 0.2 b	0.9 \pm 0.1 b
2	1.9 \pm 0.1 a	1.6 \pm 0.0 ab	1.2 \pm 0.0 a	1.1 \pm 0.0 ab
32	1.7 \pm 0.0 a	1.7 \pm 0.1 ab	1.1 \pm 0.1 a	1.1 \pm 0.0 a
64	1.9 \pm 0.1 a	1.9 \pm 0.1 a	1.2 \pm 0.1 a	1.2 \pm 0.1 a
128	1.9 \pm 0.1 a	1.8 \pm 0.1 ab	1.2 \pm 0.1 a	1.1 \pm 0.1 a

Influence of Al and B on callose formation

There was no difference in callose formation in the root tips between B treatments (Fig. 1). This indicates similar Al-injury of the root tips despite different H_3BO_3 supply. Combining similar callose formation and no difference on root elongation among a range of B supply (2-128 μM) in the presence of Al as well as in the absence of Al, it seems likely that B has little effect on Al toxicity in maize.

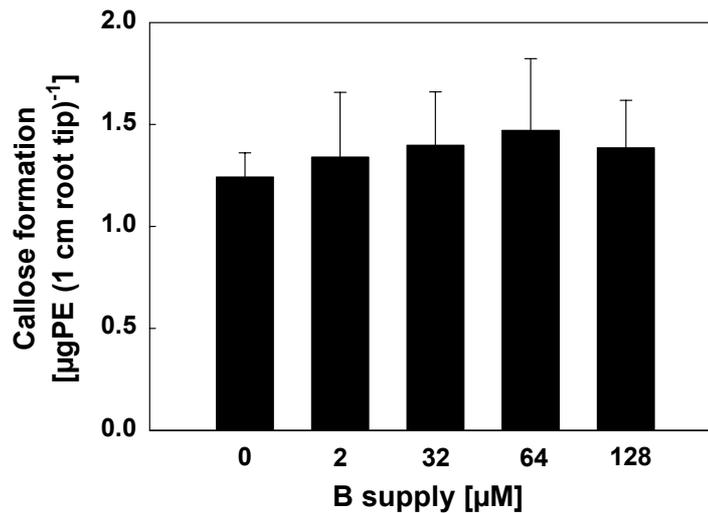


Figure 1. Influence of Al on callose formation of maize cv Lixis as affected by different B supply. Plants were grown in 500 µM CaCl₂ solution with various B supply for 48 h and were then treated with 25 µM Al for 11 and 24 h in the presence of different concentrations of B. Error bars indicate standard deviations of the mean of three replicates.

In order to confirm the above results, another set of experiment with low, medium and high B supply was conducted. In this experiment callose content, root elongation, Al and B content were determined from the same set of plants.

Effect of Al and B on root elongation

High B supply (nominal 128 µM) in the nutrition solution had a slightly negative effect on root growth, which indicated B toxicity (Fig. 2). After 12 hours Al treatment, there was clear root growth reduction, independent of B treatment. Zero B treatment did not show B deficiency because the deionized water contained about 2 µM B in this experiment. No Al/B interaction on the root elongation was found. This observation confirmed the previous results.

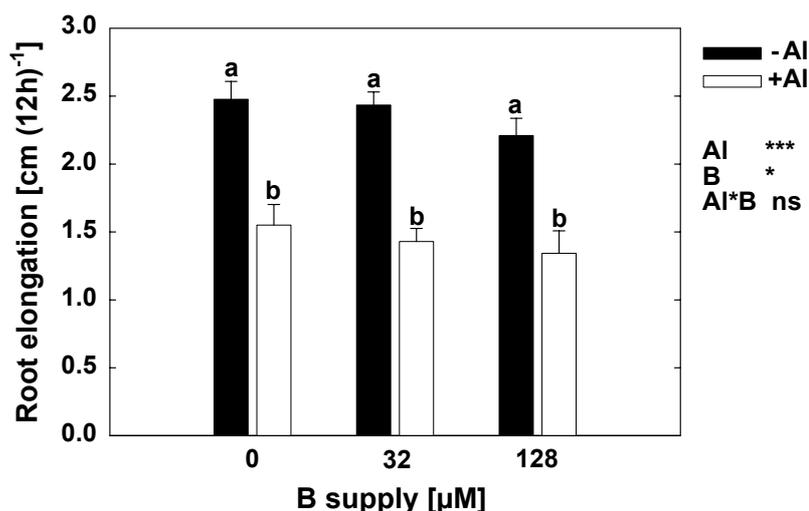


Figure 2. Effect of Al and B on root elongation of maize cv Lixis. Plants were grown in 500 μM CaCl_2 solution with various B supply for 48 h and were then treated with or without 25 μM Al for 12 h in the presence of different concentrations of B. Error bars indicate standard deviations of the mean of four replicates, each replicate consisted of 10 plants. Significant differences between mean values are indicated by different letters at the $p < 0.05$ level (Tukey test), $n = 5$. * and *** indicate significance at the $p < 0.05$ and 0.001 level according to the F test. ns = non significant.

Effect of Al and B on callose formation, Al and B contents in root tips

Aluminium strongly induced callose formation in the root tips, but there was no difference between B treatments (Fig. 3A). Also, B treatment did not affect the accumulation of Al in the 1 cm root tips of plants treated with 25 μM AlCl_3 (Fig. 3B). The B content in the root tip was extremely low compared to the Al content. There was a significant B effect on the B content in the root tip (Fig. 3C), but no difference was observed between Al treatments. For all three parameters presented in Fig. 3, no Al and B interaction existed.

In conclusion, no Al/B interaction was observed in maize in hydroponic culture under my experimental conditions and there was no evidence that boron supply could reduce aluminium toxicity.

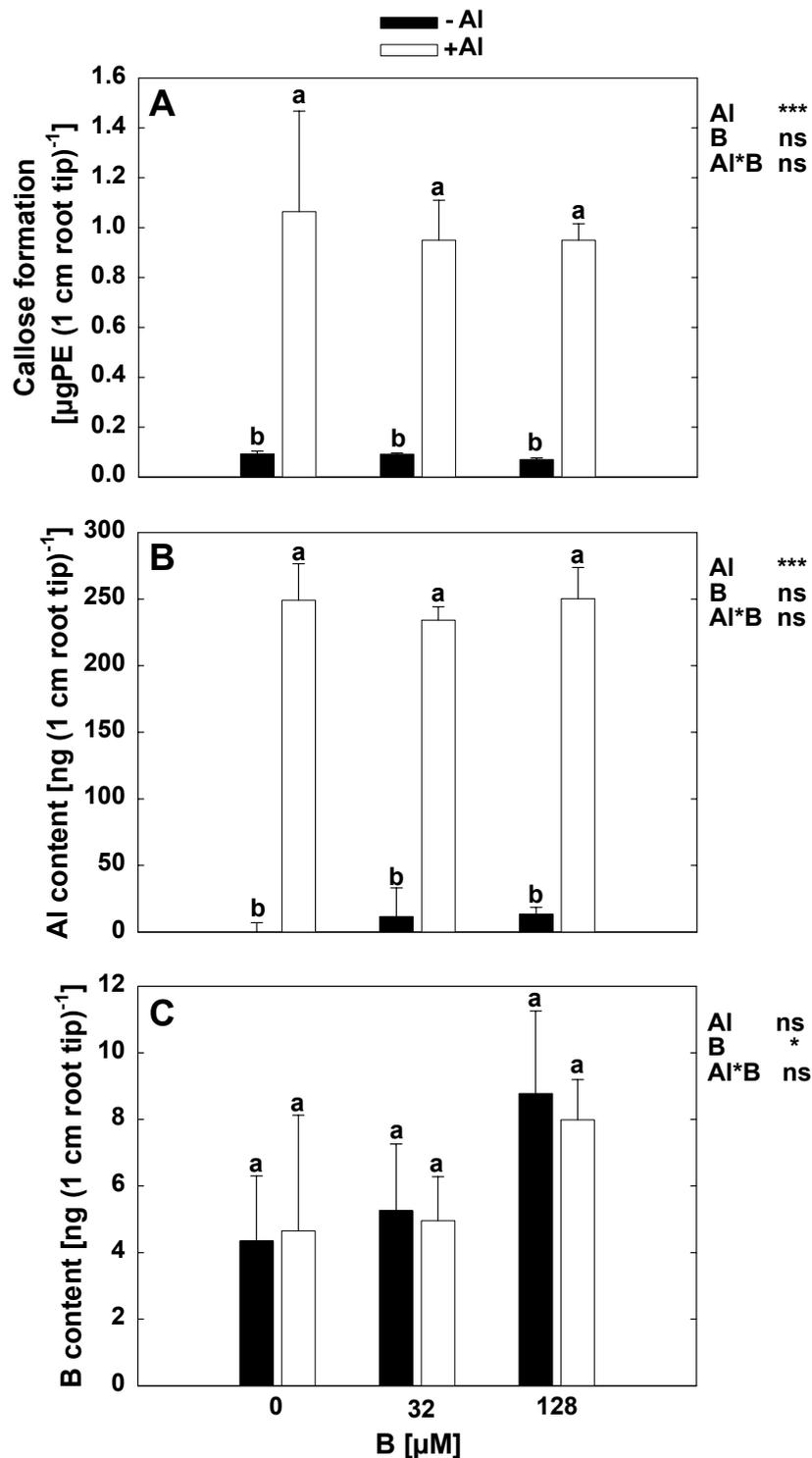


Figure 3. Effect of Al and B on callose formation (A), Al (B) and B contents (C) in the 1 cm root tips of maize cv Lixis. Plants were grown in 500 μM CaCl₂ solution with various B supply for 48 h and were then treated with or without 25 μM Al for 12 h in the presence of different concentrations of B. Error bars indicate standard deviations of the mean of four replicates. Significant differences between mean values are indicated by different letters at the $p < 0.05$ level (Tukey test). * and *** indicate significance at the $p < 0.05$ and 0.001 level according to the F test. ns = non significant.

Discussion

Aluminium toxicity and boron deficiency exhibit quite similar symptoms. Both stresses inhibit cell elongation (Horst, 1995; Brown et al., 2002) and lead to a similar morphological appearance of the roots. This includes reduced root growth, root tip swelling and abnormal cell expansion. Despite the fact that Al toxicity and B deficiency induce similar morphological changes their binding and function in the cell wall is supposed to be different.

The basis for possible Al/B interactions

Al is assumed to bind to negative charges in the cell wall, which in grasses consist of galacturonic acids, which are found in pectin, and glucuronic acids, which are found in glucuronoarabinoxylans (GAX) (Carpita, 1996). B on the other hand forms ester bonds between sugars in pectin, probably with apiose. The direct evidence for a role of B in plant growth and function is the borate ester cross-linking of the cell wall pectic polysaccharide rhamnogalacturonan II (RGII), which is required for growth and development of flowering plants. Even though the mode of action for these two elements is different, Al toxicity and B deficiency induce quite similar physiological changes.

Changes in the cytoskeleton are similar. Yu et al. (2001a) showed an increase in tubulin and actin due to B deficiency. Sivaguru et al. (2003) demonstrated that Al toxicity increases the depolymerisation of microtubules which will lead to more unpolymerised tubulin. Both stresses lead to a decrease in membrane fluidity (Vierstra and Haug, 1978; Chen et al., 1991; Ferrol et al., 1993). According to Bennet et al (1985b) a disturbance of vesicle transport is an early effect of Al. This is interpreted as a disturbance of membrane transport functions and may be a consequence of altered membrane fluidity. Also for B a change in vesicle transport has been reported (Brown et al., 2002). Goldbach et al. (2001) assumed an important role for B in the secretion of cell-wall material. Yu et al. (2002) showed that after short-term B deprivation endocytosis of cell-wall pectins was inhibited. It had been shown by Baluska et al. (2002) that under non-stressed conditions these cell-wall pectins are re-internalized after in muro deesterification. Since it has also been established that Al sensitivity in

maize can be modulated by the cell-wall pectin-content and that the degree of demethylation is of special importance (Schmohl and Horst, 2000), these findings offer an explanation for a possible Al/B interaction.

Based on these facts we developed the hypothesis that there are several links between B and Al in the cell wall. First, a cell wall sufficiently supplied with B will have a reduced porosity as compared to a cell wall deficient in B. A reduced pore size could restrict the mobility of Al. In addition, the ester bond between B and pectin provide a negative charge in the cell wall. A binding of Al to this negative charge may be less harmful to cell growth than binding of Al to the carboxylic groups of galacturonic and glucuronic acid. If B deficiency leads to an increase in pectin content and especially demethylated pectin, probably sensitive binding sites for Al would be provided.

Uncertainties concerning Al/B interactions

The aim of this study was to investigate direct effects of B on Al resistance in maize. Using root elongation, callose formation, and Al content of the root tips as parameters, no ameliorative effect of B on Al toxicity could be observed. My results are in line with other experimental approaches, where grasses were used as experimental plants.

Taylor and Macfie (1994) conducted extensive experiments on an Al-sensitive variety of wheat in solution culture and found no evidence that B was capable of ameliorating Al toxicity. They tested two alternative hypotheses regarding potential mechanisms of B amelioration of Al toxicity: the ameliorative effect could result either from alleviation of an Al-induced B deficiency or from antagonistic effects of excess B on Al toxicity, but neither could be proven to be true for wheat. More recently, results from Reid and Stangoulis (2000) also showed no evidence that supplemental B ameliorated Al toxicity in wheat. In contrast the results from Lenoble et al. (1996a, 1996b) using dicotyl plants support the hypothesis of an alleviation of an Al-induced B deficiency. They suggested the possibility that Al normally inhibits root growth by inducing B deficiency since higher B concentrations alleviated root growth inhibition and associated cellular damage caused by Al.

Apart from the links between B and Al in the cell wall, on which I based my hypothesis it has to be considered that there are also some fundamental differences in the effect of B deficiency and Al toxicity in roots. Effects of Al on root cell ultrastructure depend on the type of tissue, on the developmental stage and, particularly on the position of the cell with respect to the source of the toxic ions. Almost regularly the cells at the root-cap periphery, the cells of the root epidermis and the outer cortex undergo more drastic changes than the cells of the inner cortex and central cylinder (Čiamporová, 2002). B deficiency on the other hand affects almost all cells in the root tip, cell growth is abnormal, and under severe deficiency the apical meristem is absent (Dell and Huang, 1997). The cell-wall swelling induced by B deficiency is due to a lack of cross-linking of RG II by borate ester, not to an increase in density of the cell wall (Ishii et al., 2001). The cell-wall swelling of Al toxicity is a secondary effect, which is preceded by a reduction in root growth, and is due to an increase in pectin, hemicellulose and cellulose content (Le Van et al. 1994).

Aside from these basic differences in the effects of Al toxicity and B deficiency, some experimental conditions have to be considered to explain the results obtained. Al could increase the demand for B (Fleischer et al. 1998), but my lowest B supply may already have been sufficient to meet even an enhanced demand. Under my experimental conditions a B supply below 2 μM was not reliably possible because of variation in the B concentration of the deionised water used for the nutrient solution.

The facts that B amelioration of Al toxicity has only been reported for dicots so far may be related to differences in cell wall composition between dicots and grasses. In dicots pectin represents 30% – 40% of the cell-wall material and is the main provider of negative charges. In grasses pectin is only a minor constituent of the cell wall. Negative charges in grass cell-walls are found in pectin and in GAX, with GAX being the main source of negative charges. These differences in pectin content are also the reason for the differences in B demand of dicots and grasses. The low pectin content of grasses and the negative charges provided by GAX are probably the main reason for the conflicting results on B/Al interaction. It can be assumed that the same interaction between B and Al is taking place in grasses as in dicots, but because in grasses cell-wall properties like elasticity and pore size are less

determined by pectin. So the possible B effects on Al toxicity, if they exist, will be much more difficult to measure.

In conclusion, there is theoretical evidence for an interaction between B and Al and there are several reports documenting an ameliorative effect of B on Al toxicity. But in these positive reports plants with a high demand for B were used. All reports dealing with grasses, including my experiments with maize, could not find a positive interaction between B and Al. Therefore it can be assumed that the overall effect of B in grasses is too weak to have any significant influence on the expression of Al toxicity in grasses.

Chapter 3

Aluminium rhizotoxicity in maize (*Zea mays* L.) grown in solutions with Al^{3+} or $\text{Al}(\text{OH})_4^-$ as predominant Al species

Abstract

The rhizotoxicity of aluminium for the Al-sensitive maize cultivar Lixis in low-pH solution with Al^{3+} and in high-pH solution with $\text{Al}(\text{OH})_4^-$ as the main Al species, respectively, was studied. Aluminium reduced root growth to similar levels at pH 8.0 and pH 4.3 although the monomeric Al concentration in the pH 8.0 solutions was four times lower than in the pH 4.3 solutions. After 12 h of Al treatment, Al contents of the 1 cm root apices of plants grown in solution at pH 8.0 were much higher than that at pH 4.3. However, in contrast to pH 4.3, Al induced callose formation in the root apices only marginally, and root-tissue integrity was better maintained at pH 8.0. The largest fraction of the root-tip Al was recovered in the cell-wall fraction independent of the culture solution pH. A lower percentage of Al was recovered in the acid-wash and base-wash solutions but a higher percentage in the symplastic sap fraction in the root tips grown at alkaline pH. A sequential extraction of the isolated cell-wall material with increasing KOH concentrations suggests that most of the cell-wall Al was precipitated $\text{Al}(\text{OH})_3$ in root tips exposed to Al at pH 8.0. This can be explained by a low pH in the root apoplast at pH 8.0. I interpret my results as circumstantial evidence that at bulk solution pH 8.0 the maintenance of an acidic apoplast leads to the formation of cationic Al hydroxyl species and $\text{Al}(\text{OH})_3$ inducing root-growth inhibition but less plasma-membrane and cell damage than Al^{3+} dominating at low pH.

Keywords: Aluminium rhizotoxicity, aluminium species, maize

Introduction

Aluminium toxicity has been well documented under acid soil conditions, where Al^{3+} is the most abundant monomeric Al species leading to rhizotoxicity in plants, and is generally believed to be the most toxic form (see review Delhaize and Ryan, 1995; Matsumoto, 2000). However, Al toxicity is not only a plant growth and yield-limiting factor on acid soils, Al toxicity has also been reported in alkaline soils amended with alkaline fly ash (Rees and Sidrak, 1955; Jones, 1961) and bauxite residue (Fuller and Richardson, 1986). Also, in agreement with these observations Al rhizotoxicity has been clearly demonstrated in hydroponic culture with pH values adjusted to >8.0 (Fuller and Richardson, 1986; Kinraide, 1990; Eleftherios et al., 1993; Ma et al., 2003).

The aluminate ion ($\text{Al}(\text{OH})_4^-$) is the dominant Al species in alkaline Al solutions (Martin, 1988). But it is not clear whether the aluminate ion is the Al species leading to rhizotoxicity in the alkaline pH range. Eleftherios et al. (1993) observed aluminate-induced changes in morphology and ultrastructure of the roots of *Thinopyrum junceum* grown in nutrient solution at pH 10. In a more recent study Ma et al. (2003) presented evidence that wheat plants were significantly inhibited in growth when Al was present at a concentration of about 1 mg L^{-1} in soil solutions with a pH greater than 9. In his study addressing the rhizotoxicity of the aluminate ion, Kinraide (1990) hypothesized that aluminate was non-toxic and that the inhibition of root elongation by Al was attributable to the formation of the metastable polynuclear hydroxy-aluminium complex (Al_{13}) postulated to have formed in the free space of the roots. This is in agreement with the conclusions drawn by Poléo and Hytterød (2003) from the study of the effect of Al on Atlantic salmon in alkaline water that the toxicity of the aluminate ion is low, particularly lower than the corresponding toxicity of cationic Al hydroxides.

Although much progress has been made during recent years, the mechanisms of Al-induced inhibition of root elongation and Al resistance are still not well understood (Taylor, 1991; Kochian, 1995; Delhaize and Ryan, 1995; Matsumoto, 2000; Kochian et al., 2002). Particularly the relative importance of symplastic versus apoplastic lesions of Al toxicity remains a matter of debate. Rengel (1996) and especially Horst (1995) focused their attention on the role of

the apoplast in Al toxicity regarding short-term inhibition of root elongation by Al. This hypothesis is supported by experimental evidence: root Al injury can be modulated by the negative charge of the cell walls (Schmohl et al., 2000; Schmohl and Horst, 2000), apoplastic flow of solutes is inhibited by Al (Schmohl and Horst, 2002), cell walls are the main sites of Al accumulation (Marienfeld et al., 2000). Also, Al-induced callose formation, which is a most sensitive response of root apices to short-term Al (Wissemeier et al., 1987; Horst et al., 1997; Sivaguru et al., 1999), can be best explained by an interaction of cationic Al species with the plasma membrane.

The comparison of Al toxicity at low (predominant Al species in solution $\text{Al}(\text{H}_2\text{O})_6^{3+}$ (Al^{3+})) and high pH (predominant Al species $\text{Al}(\text{OH})_4^-$) appeared to us particularly suited to clarify the role of the apoplast versus the symplast in Al toxicity because of the expected contrasting behaviour of cationic and anionic Al in the root apoplast in spite of the expected confounding chemical processes in the root apoplast predicted by Kinraide (1990). In the present study I focused my work on the difference between low-pH and high-pH solutions on Al uptake and distribution in the root apices, and on short-term Al rhizotoxicity as reflected by inhibition of root elongation and induction of callose formation.

Materials and methods

Plant material and growth conditions

Seeds of an Al-sensitive maize cultivar Lixis were germinated between moist filter-paper rolls for three days in the darkness. Uniform seedlings were transferred to plastic pots containing 18 L of culture solution with 500 μM CaCl_2 and 8 μM H_3BO_3 . One day after transplanting, the pH of the culture solution was adjusted stepwise to the treatment target pH within 24 hours. Then the plants were exposed to 0 or 50 μM AlCl_3 for up to 36 hours. The solution pH was maintained at the target pH \pm 0.1 by adding 0.1 M KOH or 0.1 M HCl.

Since the culture solution was not well-buffered, plants grown at pH higher than 7.0 decreased the pH by releasing H^+ . Thus, in order to keep the solution pH constant 0.1 M KOH was added to 18 L culture solution every 20 minutes. At low pH, plants tended to increase solution pH slightly, which was corrected by the addition of 0.1 M HCl. In order to compensate for the K^+ input in the high pH treatments by KOH addition for pH adjustments, the plants at low solution pH were supplied with equal amounts of K^+ by adding 0.1 M KCl. The pH changes over time and acid or base addition was monitored and the input of acid and base to each pot was recorded during the whole experiment. All experiments were conducted in a growth chamber under controlled environmental conditions of a 16/8 h day/night cycle, 30/27°C day/night temperature, 75% relative air humidity, and a photon flux density of 230 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photosynthetic active radiation at plant height.

Analysis of monomeric Al concentration in nutrient solution

After treatment, the culture solutions were filtered immediately through 0.025 μm nitrocellulose membranes. Monomeric Al (Al_{mono}) concentrations were measured colorimetrically using the aluminon method according to Kerven et al. (1989).

Root length measurement

After treatment, the whole root system from 12 plants was scanned and total root length was measured using the software WinRHIZO image analysis (WIN MAC, Regent Instruments Inc, Quebec, Canada).

Root sample collection

After treatment, plant roots were rinsed with deionised water, 1 cm root tips were excised using a razor blade, stored at 4°C for Al analysis or frozen immediately in liquid nitrogen for callose determination.

Fractionation of Al in root tips

For the fractionation of Al in the root apices, 20 freshly excised 1 cm root tips of 20 seedlings were incubated in 3 ml of 0.1 mM HCl (acid washed) or in 0.1 mM NaOH (base washed) for 30 min, then rinsed with 2 ml of the same acid or base solution. After the incubation in the acid wash solution (AWS) or base wash solution (BWS) the root apices were frozen at -20°C overnight. Symplastic sap (SS) was recovered from the frozen-thawed samples by centrifugation at 3000g at 4°C for 15 min. The residue was transferred to 2 ml Eppendorf vials and 1 mL of 95% ethanol was added. Then the sample was homogenized with a Mixer mill (MM200, Retsch, Germany) at a speed of 30/s for 30 min. After centrifugation, the supernatant and pellet were separated, the pellet was washed again with ethanol followed by a second centrifugation. The two supernatants were combined and referred to as the ethanol wash solution (EWS). Cell sap and EWS together represented the symplast fraction and the pellet represented the cell-wall material (CW).

Extraction of Al from cell wall

Aluminium was extracted from the cell wall on a Millipore filtration unit by a sequential procedure using solutions of 0.10, 0.25, 0.50, 1.0, 10.0 mM KOH, each for 10 min. After the KOH solution was acidified by HNO₃, Al concentration

in the KOH solution was determined by ICP-OES (Spektro Analytical Instruments, Kleve, Germany).

Aluminium quantification

For Al analysis, the root tips or different fractions of root tips were wet digested with ultrapure concentrated HNO₃ at 135°C for 35 min in a microwave oven (MLS-ETHOS plus, Mikrowellen-Laborsystem, Leutkirch, Germany). After dilution with ultrapure water, Al concentrations in the solutions were quantified by ICP-OES (Spektro Analytical Instruments, Kleve, Germany).

Callose quantification

Three 1 cm root tips were homogenized in 500 µL 1 M NaOH for 2 min at a speed of 20/s with a mixer mill (Retsch MM 200, Haan, Germany). After homogenization, another 500 µL 1 M NaOH was added and callose was extracted for 30 min at 80°C in a water bath. Callose was quantified fluorometrically (Hitachi f2000, Hitachi, Tokyo, Japan; excitation 393 nm and emission 484 nm) according to Köhle et al. (1985), using aniline blue as colour reagent. Pachyman (Calbiochem, Deisenhofen, Germany) was used as calibration standard. Hence, callose content was expressed as pachyman equivalents (PE) per root tip.

Apoplastic sap collection and apoplastic pH measurement

For the apoplastic pH measurements, 5-days-old seedlings were grown in culture solution with different pH, with or without Al supply for 2 hours. 2 cm root tips from the primary roots or the thickest seminal roots were excised at 4°C. Excised root tips from each treatment were washed in pre-cooled (4°C) basic solution (Al-free) with the same solution pH as the treatment. The apoplastic sap of the root tips was collected by centrifugation, according to the method described by Yu et al. (1999) with some modifications. Briefly, about 30 root tips were arranged in a filter unit (Millipore Ultrafree-MC, 0.45 µm) with the cut ends facing down. The wash solution retained between adhering root tips was collected by centrifugation at 600g at 4°C for 5 min. Thereafter, the apoplastic

sap was collected by centrifugation at 3000g at 4°C for 15 min. The pH in the apoplastic sap was measured by a microelectrode (MI129, ISFET-pH-Electrode, Mettler-Toledo Analytical, Schwerzenbach, Switzerland).

Morin staining of Al in root tip

Aluminium in the root tissue was localized by staining with morin. After 8 h Al treatment, 2 cm root tips from each treatment were excised and washed in basic solution (Al-free) with the same solution pH as the treatment. Free-hand sections from the 1-3 mm zone behind the root apex were stained with 25 µM morin (pH 5.6) for 30 min at room temperature. After washing in distilled water, the sections were observed under a fluorescence microscope (excitation filter 395-440 nm, barrier filter 470 nm). Images were taken by a digital camera (Sony, DSC-S85) and then exported to Adobe photoshop 5.0.

Results

Effect of solution pH on monomeric Al concentration in culture solution

The monomeric Al concentration (Al_{mono}) in the culture solution was measured 12 h (Fig. 1A) and 36 h (Fig. 1B) after Al treatment. The Al_{mono} concentrations of the solutions with pH values adjusted to 4.3 and 10.0 were only slightly lower than the nominal Al concentration (50 µM). After 12 h Al treatment, significant losses of Al_{mono} occurred at pH 8.0 and 9.0 where only 18% and 36% of the Al added to the solution could be recovered as Al_{mono} , respectively. The losses of Al_{mono} at pH 8.0 and pH 9.0 were also observed after 36 h.

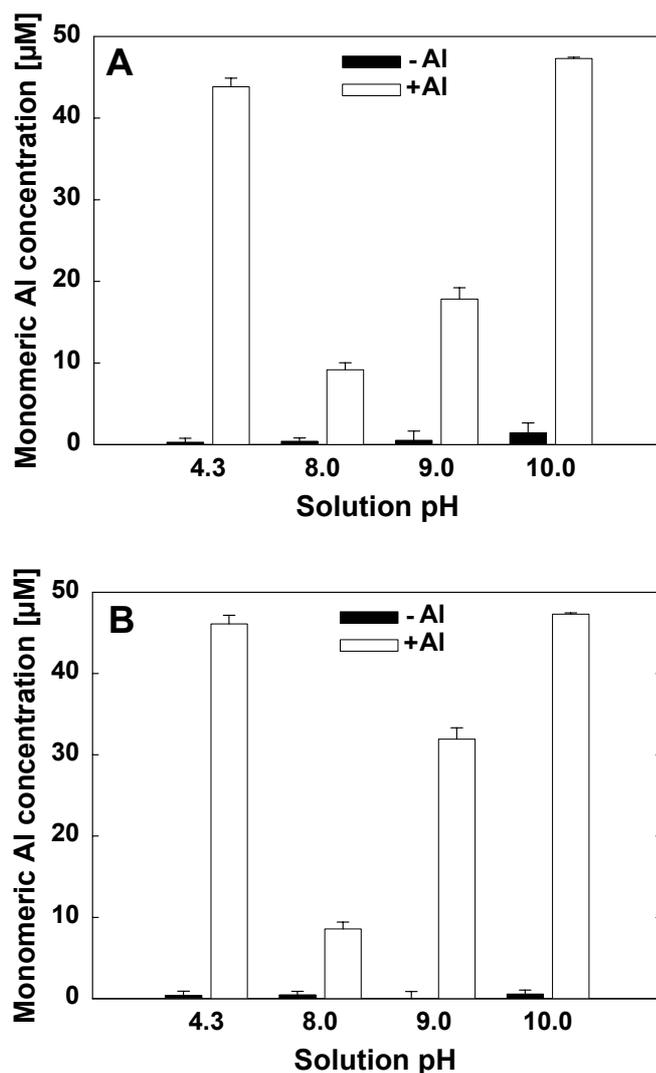


Figure 1. The effect of solution pH on monomeric Al concentration in the culture solution containing initially $500 \mu\text{M}$ CaCl_2 , $8 \mu\text{M}$ H_3BO_3 , and 0 or $50 \mu\text{M}$ Al, after the cultivation of maize seedlings for 12 h (A) or 36 h (B).

Effect of Al and solution pH on root growth

During the experiment I observed that the toxic effect of Al on root growth became progressively intensified with increasing exposure time (Table 1). After 36 h Al treatment, root growth was significantly reduced at all pH levels. Compared with the non-Al-treated controls, Al reduced root growth to similar levels at pH 4.3 and pH 8.0. At pH 9.0 and particularly at pH 10.0 the relative root growth was much less affected. This was mainly due to the severe root growth depression of the controls at these elevated pH levels.

Table 1. Total root length of maize seedling as affected by Al treatment at different solution pH. Plants were supplied with or without Al for 12 and 36 hours after adaptation to different solution pH for one day (n = 12). Relative root length: total root length of +Al treatment / total root length of -Al treatment.

Culture solution pH	Al supply (μM)	Total root length (cm plant ⁻¹)		Relative root length (%)	
		12 h	36 h	12 h	36 h
4.3	0	269	426	66	46
	50	179	197		
8.0	0	220	439	63	41
	50	139	178		
9.0	0	154	298	79	54
	50	123	160		
10.0	0	107	138	125	93
	50	134	128		

Effect of Al and solution pH on callose formation and Al content in root tip

Callose formation in the root tip was determined as an indicator of Al injury. In the root apices of plants exposed to Al at pH 4.3 for 12 h, a significant increase in callose content was found (Fig. 2A). However, the callose formation was only slightly enhanced by Al under alkaline conditions in spite of equally severe inhibition of the root growth (compare with Table. 1).

After 12 hours of Al treatment, Al contents in the 1-cm root apices of plants at pH 8.0 and pH 9.0 were much higher than that at pH 4.3 (Fig. 2B). At pH 10.0 plants accumulated much less Al in the root tips. In spite of very similar Al-induced inhibition of root growth, the differences between pH 4.3 and pH 8.0 in callose formation and Al accumulation in the root tips suggest different mechanisms of Al rhizotoxicity in maize under acidic and alkaline conditions.

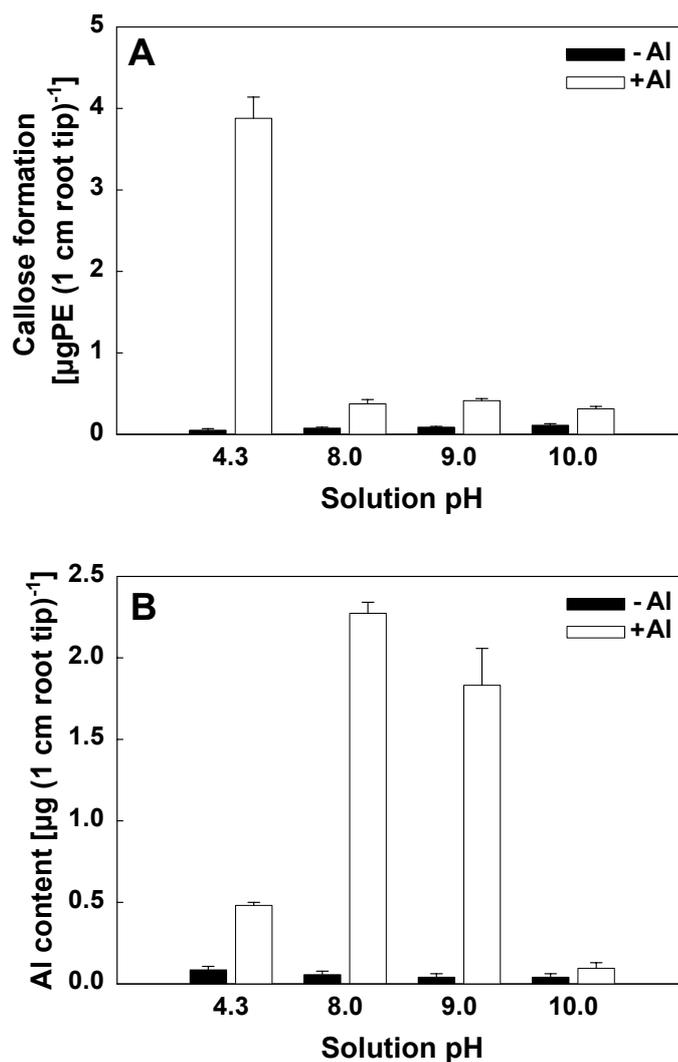


Figure 2. Effect of aluminium on callose formation (A) and Al content (B) in root tips of maize cv Lixis grown at different solution pH. pH-adapted plants were exposed to 0 or 50 $\mu\text{M AlCl}_3$ for 12 hours. Bars represent means \pm SD, $n=3$.

Fractionation of Al in root tips

In order to characterize the binding stage of Al in the root apices of plants grown at high pH compared with low pH, I subjected the root tips to a fractionation procedure. An initial washing step in acid or base aimed at differentiating between ionically bound Al and Al(OH)_3 precipitates in the root apoplast. However, there was no significant difference in Al fractionation between the root tips which were acid or base washed (Fig. 3). All Al fractions reflected the difference in total Al content between the plants treated with Al at acidic and alkaline pH: the Al content was higher at pH 8.0.

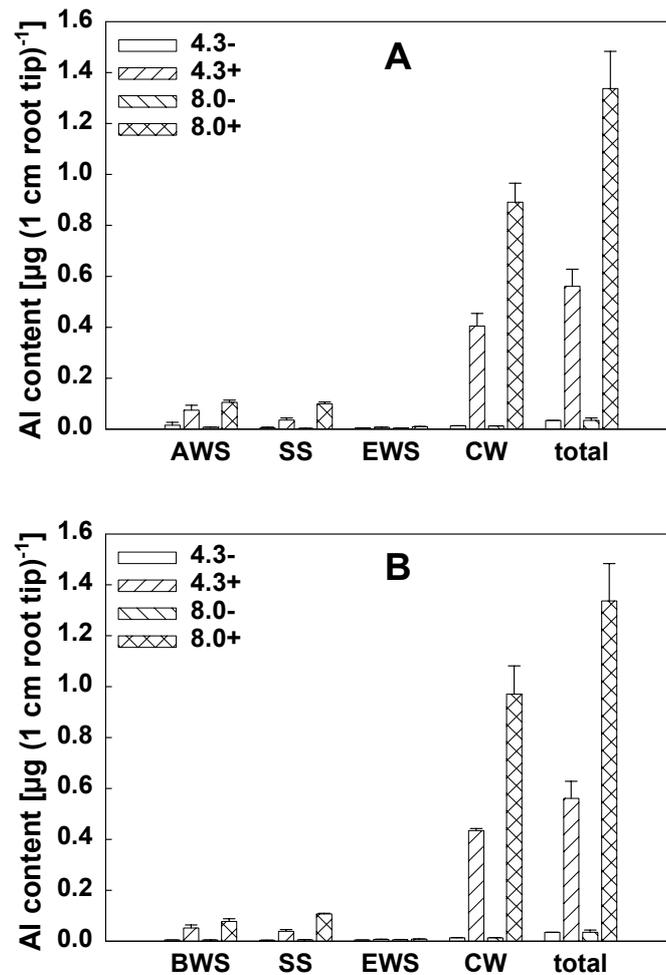


Figure 3. Aluminium content within the different fractions of the root tips of maize cv Lixis grown at pH 4.3 or pH 8.0. pH-adapted plants were exposed to 0 or 50 μM AlCl_3 for 8 hours. Excised 1 cm root tips were washed with 0.1 mM HCl (A) or 0.1 mM NaOH (B) prior to the subsequent fractionation procedure. Bars represent means \pm SD, $n=3$. AWS: Acid wash solution. BWS: Base wash solution. SS: Symplastic sap. EWS: Ethanol wash solution. CW: Cell wall.

The relative distribution of Al in different fractions of the root tips is shown in Fig. 4. The largest fraction of the root Al was recovered in the cell-wall fraction which represented 77-82% and 81-83% at pH 4.3 and pH 8.0, respectively (Fig. 4). A statistical analysis of Al distribution between pH 4.3 and pH 8.0 revealed that at pH 8.0, a significantly lower percentage of Al was recovered in both acid-wash solution ($9.5 \pm 1.0\%$ versus $14.2 \pm 1.6\%$) and in base-wash solution ($6.7 \pm 0.8\%$ versus $9.8 \pm 2.0\%$). In contrast, the Al percentage in the symplastic sap fraction was lower at pH 4.3 than at pH 8.0.

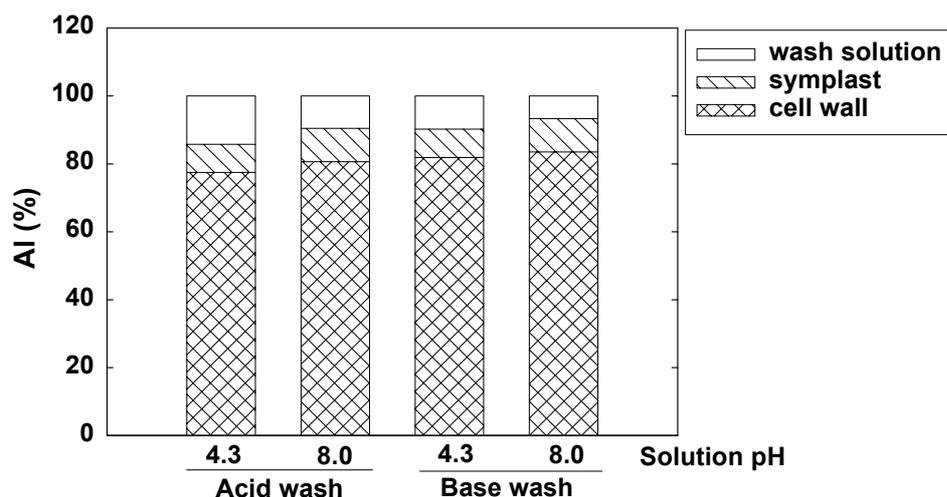


Figure 4. Distribution of Al in different fractions of the root tips of maize cv Lixis grown at pH 4.3 or pH 8.0. pH-adapted plants were exposed to 0 or 50 μM AlCl_3 for 8 hours. Excised 1 cm root tips were washed with 0.1 mM HCl (Acid wash) or 0.1 mM NaOH (Base wash) prior to the subsequent fractionation procedure.

Extraction of Al from cell wall

The speciation of Al in the cell walls of the root tips of plants grown for 8 h in presence of Al at contrasting pH was studied using a sequential extraction procedure with increasing concentrations of KOH. Hardly any cell wall Al could be solubilized by the lowest KOH concentration of 0.10 mM, independent of the pH (Fig. 5). The solubility of cell-wall Al was enhanced with increasing KOH concentration up to 10 mM for the pH 8.0 treatments. The amounts of Al released from the cell walls of plants grown at pH 4.3 increased only up to 0.5 mM KOH and then remained constant. The results indicate that at pH 8.0, the majority of cell-wall Al was $\text{Al}(\text{OH})_3$, which readily dissolved in higher concentrated KOH solution.

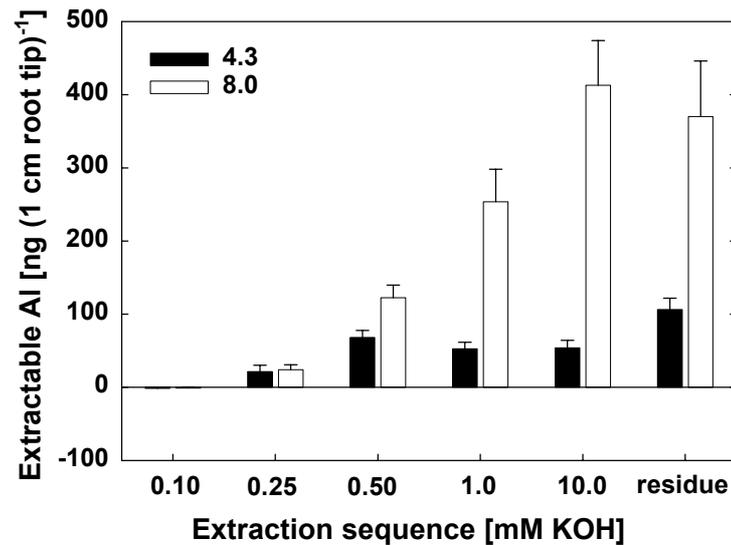


Figure 5. Aluminium extracted from cell walls isolated from 1 cm root tips of maize cv Lixis grown at pH 4.3 or pH 8.0. Cell walls of 10 root tips were sequentially extracted for 10 min each with 4 ml of 0.10, 0.25, 0.50, 1.0, and 10.0 mM KOH. pH-adapted plants were exposed to 50 μM AlCl_3 at pH 4.3 or pH 8.0 for 8 hours. Bars represent means \pm SD, $n = 5$.

Apoplastic pH

Al speciation in aqueous solution is closely related to the solution pH. Since Al is primarily localized in the root apoplast of the 1 cm root tips, the root apoplastic pH could be a crucial factor in controlling the dominant Al species in the root apoplast and thus at the outer face of the plasma membrane. The pH of the apoplastic sap of the root tips was measured as affected by pH and Al treatments (Table 2). In general, the apoplastic sap was acidic independent of the solution pH. The apoplastic pH of the root tips from plants grown at pH 8.0 was significantly higher than that at pH 4.3, with 0.3 unit difference. The apoplastic pH of root tips from plants grown in solution at pH 4.3 was very close to the solution pH. However, at pH 8.0, the apoplastic pH of the root tips was 3.4 unit lower than the pH of the bulk solution. The difference between bulk-solution pH and root-apoplast pH needs to be considered in the understanding of Al rhizotoxicity at high solution pH.

Table 2. Apoplastic pH (mean \pm SD, n = 3) of the root tips of maize cv Lixis grown at pH 4.3 and pH 8.0. pH-adapted plants were exposed to 0 or 50 μM AlCl_3 for 2 hours.

Culture solution pH	Al supply (μM)	Apoplastic pH
4.3	0	4.33 \pm 0.01
	50	4.36 \pm 0.02
8.0	0	4.67 \pm 0.05
	50	4.63 \pm 0.05

Morin staining of Al in root tip

Aluminium in the root tips was localized using morin as a stain for Al. The pattern of radial Al distribution differed between the pH treatments as well as between root-tip zones (Fig. 6). After 8 h Al treatment, all cell walls in the epidermis and the cortex were fluorescent, while the central cylinder remained unstained indicating that the endodermis with its Casparian strip represents an effective barrier for radial Al movement. Cell injury was more pronounced at pH 4.3 than at pH 8.0, especially in the 2-3 mm root zone. Many epidermal and outer cortical cells were detached at pH 4.3. At pH 8.0, although epidermal cells and outer cortical cells were intensively fluorescent, cells were not detached from the root tip. At pH 4.3, in the most Al-sensitive apical root zone, 1-2 mm from the root tip, bright and large spots were dispersed over the outer and middle cortex. This reflects the collapse of cell clusters, the reason or the consequence of the strong accumulation of Al in these cells. At pH 8.0, accumulation of Al was confined to the epidermis and the 1-2 cell layers of outer cortex. It is difficult to compare the fluorescence intensity, but in general, the fluorescence colour at pH 8.0 was more greenish than that at pH 4.3. This may reflect a greater accumulation of Al in the root tip at pH 8.0 as depicted from the chemical analysis of the total Al content in root tips (compare with Fig. 3), or a different speciation of Al species in the apoplast at pH 8.0 compared with pH 4.3. The colour of the Al-morin complex may vary with the number of positive charges of the Al species.

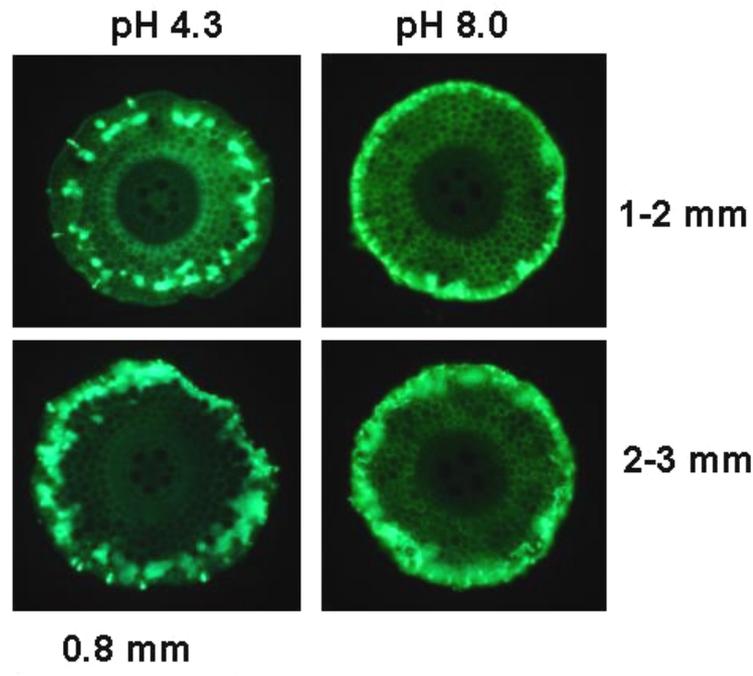


Figure 6. Fluorescence of the morin-Al complex in root cross sections (1-2 mm or 2-3 mm from the root tip) of maize cv Lixis. pH-adapted plants were exposed to 50 μM AlCl_3 at pH 4.3 or 8.0 for 8 hours. Excitation filter 395-440 nm, barrier filter 470 nm.

Discussion

Aluminium is equally toxic to maize plants in both acid and alkaline solutions based on root growth reduction (Table 1). However, considering higher Al contents in the root tips of plants grown in high-pH solutions (Fig. 2B) the Al accumulated in the roots at high pH appears to be less toxic than that at low pH. This is also indicated by results presented by Zavas et al. (1991), who studied the differential response of two populations of *Avena sterilis* L. to Al toxicity, one from an alkaline bauxite area and the other from an acid pasture area. Al contents of shoots and roots of both populations were greater at pH 10.0 than at pH 4.5, whereas better growth of both populations was observed with all Al concentrations at pH 10.0.

Al species in solution and Al toxicity

There was a good correlation between monomeric Al concentration in the culture solution and root growth reduction of the maize plants in low-pH solutions (Blamey et al., 1992). But in our high-pH solutions, root-growth reduction could not be explained by low monomeric Al concentration in the solution, especially at pH 8.0. It is possible that other Al forms were involved in the case of high-pH solutions. Polynuclear Al species Al_{13} has been proposed to form in partially alkaline solutions and was suggested to be even more toxic than Al^{3+} (Parker et al., 1989; Kinraide, 1997). In studying the formation of Al_{13} , Bertsch (1987) identified OH/Al ratio, total Al concentration, base injection rate, stirring rate as important factors. He predicted that the high pH at the point of base injection resulted in significant formation of the aluminate ion, which forms the central core of the Al_{13} polymer.

In order to maintain the solution pH constant, addition of acid or base to the culture solution is necessary, because plants grown under low pH or high pH conditions always try to increase or decrease rhizosphere pH to optimal pH. In addition, Al hydrolysis tends to bring the pH of dilute Al solution to neutral. In acid culture solution increasing the pH by base injection may result in Al_{13} formation Bertsch (1987). But to my knowledge, there is no information about Al_{13} formation in alkaline solution where the dominant Al species is the aluminate ion. Thus the assumption of Al_{13} formation in alkaline solutions through acid addition causing Al injury to plant roots in the present study remains highly speculative. However, Al_{13} formation in the acidic root apoplast cannot be ruled out (Kinraide, 1990).

Compared with the Al effect on maize plants at low solution pH, much less callose formation and dramatic high Al accumulation in the root tips of maize plants at high solution pH suggests a different mechanism of Al toxicity involved under alkaline conditions. Meanwhile, there were common features under both acidic and alkaline conditions: Al distribution among different compartments in the root tips, with the cell wall as the main location of Al accumulation under both conditions.

The relationship between solution pH and root apoplastic pH

In the present study, large amounts of base addition was necessary to maintain a high bulk solution pH, which indicated that the activity of the roots led to a release of protons into the bulk solution. Hence, the amount of proton produced by plant roots during the treatment was calculated according to the amount of base addition at pH 8.0. The rate of proton efflux was $347 \text{ nmol plant}^{-1} \text{ min}^{-1}$. Generally, H^+ released from the root would be rapidly neutralised in the bulk nutrient solution. Depending on the rate of release, the buffer power of the bulk solution, and the rate of solution agitation, a pH gradient from the root surface to the bulk solution could be substantial (Moore, 1999). Whereas in the bulk solution the pH was kept constant by the addition of KOH, a pH decrease at the root surface and even more in the root apoplast can be expected.

The pH of the bulk solution is different from the root surface and root apoplast even in hydroponic culture (Cleland, 1976; Jacobs and Ray, 1976; Pilet et al., 1983; Shabala et al., 1997; Felle, 1998; Kosegarten et al., 1999; Yu et al., 2001). By use of microelectrodes, the relationship between the pH values of the bulk medium, the root surface and the cortical apoplast was investigated in the presence of different bulk medium pH values by Felle (1998). He demonstrated that the apoplastic pH of the root tip of maize was maintained between 5.1 and 5.6. At higher bulk-medium pH the root surface pH of the first 1 cm root tip was clearly more acidic than the bulk-medium pH. At lower bulk-medium pH values the root surface pH became less acidic than the bulk-medium pH. An increase of bulk-solution pH from 4.5 to root surface pH 5.3 has also been shown with the same maize cultivar used in this study, cv Lixis, by Kollmeier et al. (2000). Similar results were obtained by Kosegarten et al. (1999) with a different technique. In their experiments the apoplastic pH in the outer cortex of root zones of maize was measured using the pH-dependent fluorescence ratio of fluorescein boronic acid. Under conditions of saturating ion concentrations, the apoplastic pH was determined along the root axis ranging from 1 to 30 mm behind the root tip. With an external solution pH of 5.0, the apoplastic pH was about 5.1 in the division zone, between pH 4.8 and 4.9 in the elongation region and about pH 4.9 in the root hair zone. At an external pH of 8.6, the difference between the external pH and the apoplastic pH was

considerably bigger, with a pH of 5.2-5.3 in all root zones. Studies determining the cell wall pH *in vivo* indicated that it may be close to 5 (Schopfer, 1989) which agrees with the observation that the majority of wall hydrolases also have an optimum pH of around 5 (Taiz, 1984).

These results from the literature are in agreement with our own measurements of the pH in the apoplastic sap, recovered from the root tips by centrifugation (Table 2). They clearly show that the plants have been able to strongly decrease the pH in the apoplast in spite of rigorous control of the bulk solution pH at 8.0. The fact that the pH was even lower than the expected optimum pH of 5.0-5.5 (see above) may be attributed to the time necessary to handle the plant tips until centrifugation. Although attempts have been made to rigorously keep the root tips at low temperature on ice, it cannot be excluded and it is likely that the highly active proton pumping from the symplast into the apoplast has continued to some extent, acidifying the apoplast more than under *in-vivo* conditions where proton was buffered by the bulk solution. We may speculate that the severe root-growth inhibition of control plants (not treated with Al) at pH 9.0 and particularly at pH 10.0 is due to the inability of the plants to acidify the root tip apoplast to the acidic pH which is necessary for optimum cell elongation (Rayle and Cleland, 1992; Cosgrove, 1998) and to avoid an increase in the cytosolic pH above the optimum (Gerendás and Ratcliffe, 2000).

The possible mechanisms of pH dependent Al toxicity

Since the Al speciation in solution is pH dependent, the pH gradient will influence the Al speciation and behaviour in the bulk solution, at the root surface, and particularly in the apoplast of the root tip. Based on the above discussion I elaborate in the following a hypothesis to explain the possible reactions involved in Al toxicity in low and high-pH solutions, where different pH gradients build-up between the medium and root apoplast. In my experiments, under conditions of the low and rather stable solution pH of 4.3, Al^{3+} is the predominant Al species. As a trivalent cation, Al^{3+} is possibly responsible for callose formation. In the case of the high-pH solution, where the pH changes from 8.0 to around 5.0 in the apoplast, protonation of $\text{Al}(\text{OH})_4^-$ takes place and results in a mixture of Al species including $\text{Al}(\text{OH})_4^-$, $\text{Al}(\text{OH})_3$, $\text{Al}(\text{OH})^{2+}$,

$\text{Al}(\text{OH})_2^+$. In this pH range, it is unlikely for Al^{3+} to occur, which could be the reason for strikingly less callose formation in the root tip under high-pH conditions.

The apoplast is negatively charged due to the acidic groups of the cell-wall materials. When the apoplast pH is elevated, dissociation of the carboxyl group of the cell-wall constituents will provide more negative charges. Therefore, the amount of positively charged Al bound to the negatively charged cell walls will be enhanced. In addition, the cell wall-bond enzyme, pectin methylesterase, which has an optimum pH of 8.0 (Goldberg, 1984), promotes the generation of negative charges in the cell wall. Both factors could contribute to enhanced binding of Al in the cell walls and thus higher Al contents in the root tips of plants grown in Al solutions with high solution pH. Another possible reason for higher Al contents in the root tips at high pH is (in addition to the precipitation of $\text{Al}(\text{OH})_3$, see discussion below) that the average positive charge density of the cationic Al species ($\text{Al}(\text{OH})^{2+}$ and $\text{Al}(\text{OH})_2^+$), which are expected to predominate at pH 5 in the apoplast, is less than that under low pH conditions with Al^{3+} as the main species. Assuming the negative charge density of cell walls as constant, more Al will bind to the root-tip apoplast of plants grown in high-pH than in the low-pH solution.

There is little doubt that binding of Al to the pectic matrix has substantial effects on the physical properties of the cell wall such as extensibility and permeability (Horst, 1995). Aluminium not only rapidly affects cell-wall but also plasm-membrane characteristics, because cell-membrane surfaces are usually negatively charged. The extent to which Al^{n+} is bound depends on the cation exchange capacity of the roots resulting from negative charges carried on pectin, proteins and phospholipids in the cell wall and on the plasma membrane (Horst, 1995). But the binding strength of different Al species to negative charges in the apoplast is not necessarily the same. Such differences may account for the differences in phytotoxicity of Al species in the root tip. Based on the similar root growth reduction but different Al content in the root tip at pH 4.3 and pH 8, I assume that Al species with higher positive charges, mainly Al^{3+} , is more effective in reducing cell-wall extensibility. A higher percentage of Al remaining in the residue fraction after KOH extraction at pH 4.3 compared to pH 8.0, indicates a stronger binding of Al to the cell walls under acid conditions

(Fig. 5). Since very little callose formation was induced by Al in the root tips at high pH in spite of high root Al contents, it appears that Al species with low positive charge can not effectively trigger callose formation. Al^{3+} appears to be more toxic in the light of membrane impairment, because cell death in the middle cortex was observed as early as after 4 h Al treatment at pH 4.3, but not at pH 8.0 (data not shown). Even after 8 h Al treatment, no such injury was observed at pH 8.0, whereas at pH 4.3 root injury in the epidermal and outer cortex was intensified as visualised by severe disruption of root-tissue integrity (Fig. 6). Based on a similar response of roots to Al and La to cation ameliorative treatments, Kinraide et al. (1992) concluded that Al^{3+} , rather than $\text{Al}(\text{OH})_2^+$ or $\text{Al}(\text{OH})_2^+$, is the principal toxic mononuclear Al species. Moore (1999), however, suggested that it is a hydrolysis product of Al rather than Al^{3+} that is responsible for inhibiting root growth. My results comparing Al toxicity at low pH and high pH suggest that both Al^{3+} and the hydrolysis products of Al are toxic to plant root in reducing root elongation. The fact that at pH 8.0 root-growth inhibition by Al was as pronounced as at pH 4.3 but membrane damage and tissue disintegration was much more intense at pH 4.3, corroborates earlier suggestions (Horst, 1995) that Al-induced inhibition of root elongation can be explained merely by apoplastic lesions.

There is no doubt that the decrease of pH from 8.0 in the bulk solution to 5.0 in the apoplast will lead to massive precipitation of $\text{Al}(\text{OH})_3$ in the root apoplast. This is corroborated by the fractionated extraction of the cell walls (Fig. 5) where Al is solubilized particularly at higher KOH supplies which readily solubilize freshly precipitated $\text{Al}(\text{OH})_3$ as revealed by parallel batch experiments (data not shown). At low bulk solution pH, the formation of $\text{Al}(\text{OH})_3$ in the root apoplast (pH 4.3) is unlikely. The release of Al from the cell-wall material at low pH appears to reflect the release of Al from negative binding sites through comparatively high K^+ concentrations (Grauer and Horst, 1992) and, at higher KOH concentrations, by partial solubilisation of cell-wall pectins (Coimbra et al., 1996). The precipitation of $\text{Al}(\text{OH})_3$ may be regarded as a detoxification of rhizotoxic monomeric Al species. However, it cannot be ruled out that the precipitate as discussed in relation to fish dying in acidic lakes (Spry and Wiener, 1991) acts as a physical block to the diffusion of nutrients and other solutes necessary for root growth through the apoplast.

GENERAL DISCUSSION

The last several decades have seen great progress in understanding of solution chemistry of Al and of the toxicity of Al to biological systems. It is clear now that soluble Al (mainly Al^{3+}) in the soil solution is the main toxic Al species responsible for growth inhibition in widely distributed acid soils. The better understanding of Al chemistry and the mechanism of Al toxicity helps to improve crop production in such soils. Reducing the solubility of Al is the major objective of the management of Al toxicity through agricultural practices.

Aluminium speciation and Al toxicity

Although the knowledge about Al toxicity and Al resistance has been growing fast, there are still a lot of knowledge gaps. For instance, it is well known that Al accumulates in the root apex, primarily in the root apoplast, but the form in which Al is bound is not yet understood (Haynes, 1984). The uncertainty is compounded by differences between the ionic composition of bulk solution and of the solution present in the apoplast, and by the possible formation of highly charged polynuclear Al complexes in the apoplast that might occur if high local concentration of Al and/or localized high pH create favorable conditions for Al polymerization (Rengel, 1996; Tice et al., 1992). The exact chemical speciation of Al in the cell apoplast remains elusive. The identity of Al complexes in contact with the plasma membrane and the time-course of their transfer into the cytosol are beyond the limits of current experimental techniques (Rengel, 1996). Therefore, indirect approaches were employed to elucidate the complexity of Al chemistry in the living root tissues.

My data indicate that the form of Al in the cell apoplast or outer surface of the plasma membrane is important for Al toxicity to express. The total Al content in the root tip is not the determinant factor of Al toxicity. It is rather the highly positively charged Al^{3+} which is lethal to root-tip cells. Such a conclusion is supported by the experimental evidence of Si amelioration of Al toxicity (Chapter 1) as well as by comparing Al toxicity in solution with different pH and

thus dominating Al species in solution (Chapter 3). These results clearly show that the formation of HAS and of less positively charged hydroxyl-Al species in the apoplast reduce Al toxicity. The common point of the ameliorative effect of H_4SiO_4 and OH^- , also of H_2PO_4^- (Taylor, 1991; Pellet et al., 1997) and of organic acids (Ma et al., 2001; Ryan et al., 2001; Mariano and Keltjens, 2003) could be the reduction of the apoplastic active Al^{3+} , although not necessarily reducing total apoplastic Al.

Apoplastic versus symplastic lesions of Al toxicity

Aluminium primarily affects the plant roots. Under controlled conditions in solution culture, inhibition of root elongation can be measured within hours after application of Al (see review Horst, 1995). The mechanism of Al-induced inhibition of root elongation is still not well understood. It remains a matter of debate whether the primary lesions of Al toxicity are apoplastic or symplastic (Horst et al., 1999a). Great difficulties represent the accurate separation of symplastic and apoplastic fractions, because, at present, there is no reliable quantitative method which can overcome the problem which presents a relatively large apoplastic Al pool remaining after desorption of intact root cells of higher plants (Rengel, 1996). Studies with giant cells of *Chara corallina*, where physical separation of the cell wall and cytoplasm after the Al uptake period can be achieved surgically (Rengel and Reid, 1997), is the most precise method for separation of the symplastic and apoplastic pool. The estimate of symplastic Al by this method is several orders of magnitude lower than other published values in which an important part of the cell-wall Al was attributed to the symplastic Al (Tice et al., 1992; Archambault et al., 1996; Kataoka and Nakanishi, 2001). The fractionation method applied in this study also does not exclude the possibility of overestimating the symplastic Al. Since after the plasma membrane was ruptured by freezing and thawing symplastic sap got in contact with the cell wall releasing some cell-wall Al into the symplastic sap. However, even though I overestimated the amount of symplastic Al, symplastic Al accounted for only 10% of total root tip Al (Chapter 1, Fig. 5; Chapter 3, Fig. 4) The results showed that a major site of Al accumulation was the cell wall, but

whether the Al accumulated in the cell wall exerts the most deleterious effect has not been fully elucidated. The binding stage of Al in the cell wall of the root apex was studied using a fractionated desorption procedure with BaCl_2 followed by $\text{Na}_3\text{citrate}$ as extractants (Chapter 1, Fig. 5). Considering the callose formation in the root apex, I conclude that the mobile apoplastic Al determine Al activity at the plasma membrane and thus Al toxicity. This conclusion was confirmed in Chapter 3: higher apoplastic Al precipitation in the root tips of plants in solution with $\text{Al}(\text{OH})_4^-$ as predominant Al species resulted in marginal callose formation and better root-tissue integrity.

In addition to the physical or chemical separation of the symplastic and apoplastic pools of Al, localization of Al at the cellular level could contribute to clarify Al compartmentation. By the aid of microscopy, Al in the plant roots or in the cells can be visualized. Unfortunately, the methods applied for Al localization were not sensitive enough to detect biological active Al precisely. The most common method of Al localization on the tissue level is by morin staining (Tice et al., 1992; Larsen et al., 1996; Cocker, 1997; Ahn et al., 2002; Ermolayev et al., 2003). The fluorescent dye morin was supposed to bind to biological active Al (Browne et al., 1990), but Taylor (1995) expressed his doubt that morin was capable of detecting Al which was tightly bound to the cell wall. These opinions give rise of questions such as: is the tightly bound Al in the cell wall not the biological active Al? In other words, is Al tightly bound to cell wall less harmful to cells?

The main function of the cell wall is to keep the cell shape and protect the cytoplasm. Change in cell-wall composition in response to Al may be an important strategy for cells to protect themselves. An increase in cell-wall pectin, as well as hemicellulose and cellulose, has been reported along the root axis in squash seedlings treated with Al in nutrient medium (Le Van et al., 1994). It has been suggested that a minor part of pectin is a major site of Al accumulation, the content of cell-wall pectin increased during Al treatment in nutrient solution. Hence, Chang et al. (1999) hypothesized that Al may bind to the pectin newly produced during Al treatment. In the work on identification of Al-regulated genes using cDNA-AFLP in rice, Mao et al. (2004) found that Al stress could induce the biosynthesis of lignin and other cell-wall components in

roots. There is adaptive significance for cell walls to bind and thus inactivate Al because a most sensitive physiologically functional part of a cell is the plasma membrane and the symplast. Al sequestered in the cell wall may reduce the amount of Al contacting or entering the plasma membrane. On the basis of my results (Chapter 1, 3), I propose that Al tightly bound to the cell wall and/or Al precipitated in the cell wall could have adverse effect on cell growth by reducing cell-wall extensibility and inhibiting apoplastic flow of solutes, but will not cause incipient cell death. It is rather the mobile Al (Al^{3+}) in the apoplast and/or bound to the external face of the plasma membrane that trigger the cell lesion.

Al uptake into the cell triggering cell lesion can not be excluded, but at present, there is no unambiguous concept with regards to Al uptake into the symplast and resulting Al toxicity.

Kochian (1995) stated that there is no real conceptual basis for the assumption that Al binding within the cell wall is a prerequisite for Al uptake. The actual transport site for uptake of ions across the plasma membrane would be the solution phase adjacent to the outer plasma membrane surface, and there is no reason to expect that the Al fairly tightly bound to the Donnan sites within the cell wall would have a large effect on this transport pool. On the other hand, the actual state of Al speciation in the cell-free space and cell wall, just like in the soil, is permanently changing to reach its equilibrium (Haug, 1984). Certainly, loosely bound Al in the cell wall would have an effect on this transport pool.

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