RESPONSES OF SUGARCANE TO ALUMINIUM TOXICITY

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original word and that I have not previously in its entirely or in part submitted it at any university for a degree.

Signature:

Date: 03 / 03 / 2006

ABSTRACT

The aims of this study were (1) to determine whether NO_3^- or NH_4^+ nutrition could influence the effect of Al on N12 and N19 sugarcane plants grown in sand or hydroponic medium and (2) to assess whether the different root environments of sand and hydroponically cultured plants could influence the effect of Al on N12 and N19 sugarcane plants supplied with NO_3^- or NH_4^+ nutrition. N12 and N19 sugarcane was grown in sand and hydroponic culture with and without Al and with either NO_3^- or NH_4^+ as N source. Biomass accumulation, tissue N, P and Al and root assimilation of $^{14}CO_2$ supplied to the root system were measured.

Both N12 and N19 sugarcane plants were found to be relatively Al tolerant (tolerating up to 1 mM Al). This lack of effect of Al on plant growth might be due to amelioration of Al-toxicity in sugarcane by the adequate supply of carbon skeletons from the C_4 photosynthetic pathways of sugarcane. The supply of carbon skeletons may enable both cultivars to exude large amounts of organic acids into the rhizosphere, which confers a dual advantage to these plants. Organic acids can form soluble complexes with Al thus preventing its entry into the roots and can form soluble complexes with nutrients (cations), which makes some nutrients (e.g. P) more available for plant uptake. The availability of carbon in the root system is dependent on the N source. Increased growth of Al treated plants supplied with NH_4^+, relative to those grown on NO_3^-, might be due to the capacity of C_4 photosynthesis to meet the needs for both the assimilation of NH_4^+ into amino acids and the synthesis/excretion of organic acids for Al-detoxification. The fact that growth was improved with NH_4^+ and Al may indicate that NH_4^+ and Al cations compete for access to the root tissue resulting in an increase in root activity, organic acid exudation and nutrient uptake.

It was postulated that sand-grown plants would be less influenced by Al than hydroponic plants because organic acids can form a protective sheath that shields the root apex from the toxic Al cations in sand due to the relative lack of mobility of the soil solution. However, Al increased the growth of NH_4^+-fed hydroponically grown plants more than that of NH_4^+-fed sand grown plants. Thus we did not find evidence to support our expectation that the roots of the hydroponically grown plants would be

more exposed to Al due to nutrients and organic acids being uniformly distributed in the growth solution compared to sand grown plants.

OPSOMMING

Die doelwitte van die studie was om te bepaal of (1) NO_3^- of NH_4^+ voeding die effek van Al beïnvloed op N12 en N19 suikerriet plante wat gegroei is in sand of hidroponiese medium en (2) te bepaal of die verskillende wortel omgewings van sand en hidroponiese gekultureerde plante die effek van Al op N12 en N19 suikerriet plante voorsien van NO_3^- of NH_4^+ voeding. N12 and N19 suikerriet plante was gegroei in sand en hidroponiese kulture met en sonder Al en met eerder NO_3^- of NH_4^+ as N bron. Biomasse ophoping, weefsel N, P en Al, wortel assimilasie van $^{14}CO_2$ gevoorsien aan die wortel was gemeet.

Beide N12 en N19 suikerriet plante was bevind om relatief Al tolerant (tolerantsie tot 1 mM Al) te wees. Die geringe effek van Al op plant groei mag toegeskryf word aan die verbetering van Al-toksisiteit in suikerriet plante deur die genoegsame voorsiening van koolstof geraamtes van die C_4 fotosintetiese paaie van suikerriet. Die voorsiening van koolstof geraamtes mag beide kultivaars toelaat om groot hoeveelhede organiese sure uit te skei in die rhisosfere, wat 'n tweedoelige voordeel gee aan die plante. Organiese sure kan oplosbare komplekse met Al vorm wat toegang tot wortel voorkom en kan ook oplosbare komplekse met voedingstowwe (katione) vorm wat party voedingstowwe (bv. P) meer geredelik beskikbaar maak vir opname. Die beskikbaarheid van koolstowwe in die wortel sisteem is afhanklik van die N bron. Verhoogde groei van Al behandelde plante voorsien van NH_4^+, relatief tot die gegroei in NO_3^- mag te wyte wees aan die vermoë van die C_4 fotosintese om die behoeftes te voorsien van beide die assimilasie van NH_4^+ in die aminosure en die sintese/uitskeiding van organiese sure vir Al-detoksifisering. Die feit dat groei verbeter het met NH_4^+ en Al mag 'n indikasie wees dat NH_4^+ en Al katione kompeteer vir toegang tot die wortel weefsel wat meebring 'n verhoging in wortel aktiwiteit, organiese suur uitskeiding en voedingstof opname.

Dit was gepostuleer dat sand-gegroeide plante minder beïnvloed word deur Al as hidroponiese plante omdat organiese sure vorm 'n beskermende skede wat die wortel punt beskerm teen die giftige Al katione in sand as gevolg van die relatiewe gebrek aan beweging van die sand oplossing. Alhoewel, Al die groei verhoog van NH_4^+-gevoerde hidroponiese gegroeide plante meer as die van NH_4^+-gevoerde sand gegroeide plante. Ons vind dus bewyse om ons verwagting te ondersteun dat die

wortels van hidroponies gegroeide plante sal meer blootgestel wees aan Al as gevolg van voedingstowwe en organiese sure wat eenvormig versprei is in die groei oplossing vergelyke met die van sand gegroeide plante.

ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
CEC	cation exchange capacity
DNA	deoxyribonucleic acid
PEP	phosphoenolpyruvate
PM	plasma membrane

TABLE OF CONTENTS

Chapter 1

1.1 General introduction

South Africa is currently rated as the 8th largest producer and 9th biggest exporter of sugarcane in the world (www.illovo.co.za). However, the crop yield and productivity of the South African Sugar Industry has reached a plateau (Van Antwerpen & Meyer, 1996). Soil degradation, in particular soil acidification and loss of soil organic matter, both recognised consequences of intensive mono-cropping, have been indicated as the main factors limiting increased crop production (Schroeder *et al.*, 1995). Soil acidification develops when basic cations are leached from soils, but can also be accelerated by farming practices such as the usage of marginal soils, poor irrigation water as well as the common practice of applying NH_4^+ fertilisers to soils. It has been predicted that the severity of soil acidification will further increase in South African regions producing sugarcane (Schroeder *et al.*, 1994).

Phytotoxic effects of Al have been studied since their experimental confirmation at the beginning of the 20th century. The reason for the interest is a wide occurrence of soils with low pH, in which Al acquires a chemical form that can be taken up by plants and have toxic effects (Ciamporova, 2002). Soils often contain toxic concentrations of Al ions (10 to 100 μM), which may have a negative impact on crop yields. Al toxicity also influences distribution of plant species in the natural environments. Therefore, the interest is concentrated on understanding the mechanisms of Al effects and, on the mechanisms by means of which a plant "protects itself" against the Al toxicity (Taylor, 1995). Concomitantly, considering the complex network of reactions and interaction would allow the definition of responses of whole plants to the presence of Al in the root zone (Bennet & Stewart, 1999).

Plant species and genotypes within species differ widely in their tolerance to Al (Foy, 1988; Taylor, 1987). However, sugarcane has been identified as a crop species adapted to acid soils and appears to be tolerant to high levels of NH_4^+ that result from inhibition of nitrification in acid soil (Foy *et al.*, 1978). Based on a reported adaptation to acid soils, tolerance to Al toxicity is also suspected. In field trials, preliminary screening of commercial varieties has indicated that the varieties N12 and N16 appear to be more tolerant of Al than NCo376, which is the reference variety

against which all others are compared. There is some evidence that the apparent tolerance of these two varieties is associated with a negative interaction at high rates of lime application. An Al saturation index value of 20% is proposed as the threshold for all varieties, except N12, above which a response to lime is likely. A tentative threshold Al threshold value of 40% is suggested for N12 (Schroeder *et al.*, 1995). Some of the latest sugarcane varieties released by SASRI are more tolerant to Al than the reference NCo376 variety (Anonymous, 1993).

Amongst the earliest symptoms of Al toxicity are the inhibition of root growth, poor root hair development and the appearance of swollen root apices (Cramer & Titus, 2001). Inhibition of root growth is very rapid with 5% inhibition of the elongation rate being recorded within 33 min in maize supplied with 20 uM Al (Llugany *et al.*, 1995). Whether or not Al toxicity is viewed as a root- or a shoot- related phenomenon, several possible mechanisms by which Al may disrupt cellular function have been proposed. These include disruption of membrane structure and function, inhibition of DNA synthesis and mitosis, inhibition of cell elongation and disruption of mineral nutrition and metabolism (Taylor, 1988a).

The resistance of plants to Al toxicity can be classified into external exclusion mechanisms and internal tolerance mechanisms (Taylor, 1988a; Kochian, 1995). The main difference between these two types is the site of Al detoxification: symplasm (internal) or apoplasm (external), (Ma, 2000). The proposed mechanisms for exclusion include immobilization at the cell wall, selective permeability of the plasma membrane, plant-induced pH barrier in the rhizospere and exudation of chelate ligands. By contrast, internal tolerance mechanisms include chelation in the cytosol by organic acid, compartmentation in the vacuoles, Al-binding proteins and evolution of Al-tolerant enzymes (Kochian, 1995; Taylor, 1991). Most of these mechanisms remain to be studied.

Recent evidence shows that organic acids play an important role in both the internal and external Al detoxification (Ma, 2000). The molecular mechanisms underlying Al toxicity are not known, but because Al forms strong bonds with oxygen–donor compounds, it can interact with multiple sites in the apoplasm and symplasm of root cells (Ma *et al.*, 2001). Organic acids are mainly produced in mitochondria through

the tricarboxylic acid or Krebs cycle and to a lesser extent in the glycarboxylic cycle. Because of the catalytic nature of the Krebs cycle, organic acids are present only in very small pools in the mitochondria and are preferentially stored in the vacuole (Lopez-Bucio *et al.*, 2000). Over a dozen Al-tolerant plant species are known to secrete organic acids from their roots in response to Al treatment (Ryan *et al.*, 2001). Succinate, citrate, oxalate, malate, oxalate, lactate and fumarate are some of commonly released organic acids that can form sufficiently strong complexes with Al to protect plant roots (Ma *et al.*, 2001).

NO_3^- is the predominant form in which inorganic nitrogen is taken up by plant roots. Once inside the cell, NO_3^- is converted to the reduced cation NH_4^+, which is utilized in amino acid biosynthesis (Lopez-Bucio *et al.*, 2000). NO_3^- nutrition results in alkalization of the root zone while NH_4^+ nutrition results in acidification (Kosegarten *et al.*, 1999), the latter possibly exacerbating Al toxicity. Although most plants can use either or both forms as a source N, NO_3^- is generally the preferred source for crop growth (Lasa *et al.*, 2001). However, excessive NO_3^- application can have detrimental effects such as contamination of ground water via NO_3^- leaching and gaseous losses of nitrogen as N_2O, a factor leading to deterioration of the ozone layer (Barker; Mills, 1980). On the other hand NH_4^+ fertilization can be a desirable source of N nutrition under certain conditions. Furthermore, NH_4^+ application would seem to be a factor in establishing best management practices since the NH_4^+ ion is not readily subject to leaching and denitrification losses (Lasa *et al.*, 2001).

The ability of organic acids to chelate and render Al non-phytotoxic is well established and it has been found that Al tolerant plants used organic acids, especially malate and citrate, to detoxify Al both internally and externally (Delhaize & Ryan, 1995; Ryan *et al.*, 1995; Jorge & Arruda, 1997; Ginting *et al.*, 1998; Ma *et al.*, 1998; Zheng *et al.*, 1998a; Zheng *et al.*, 1998b). NO_3^- uptake rates by roots of intact soybean plants are stimulated by malate, which moves down the phloem and accumulates in the root (Touraine *et al.*, 1992). Organic acids have also been found to stimulate NO_3^- uptake. The relationship between NO_3^- assimilation and organic acid metabolism has been elegantly explored in mutant plants with low nitrate reductase activity, which accumulate large amount of NO_3^- (Scheible *et al.*, 1997).

Tolerance to Al toxicity involves processes that require carbon allocation and energy thus leading to a loss in yield. A possible manipulation of carbon metabolism, especially of the root, could increase the capacity of the root to withstand the carbon drain associated with tolerance mechanisms (Lidon *et al.*, 1997). Such manipulations may optimise the productivity of resistant varieties and also facilitate the development of environmentally sound management practices for Al resistant crop production.

The purpose of this study is to investigate Al toxicity in N12 and N19 sugarcane plants using NO_3^- or NH_4^+ as a nitrogen source. This was addressed by assessing the influence of N nutrition during Al phytotoxicity on sand and in liquid, to determine whether responses on these two media differed.

Supply of NH_4^+ as a N nutrition source exacerbates Al toxicity because NH_4^+ accumulation results in acidification of the rhizosphere, while NO_3^- accumulation results in alkalization of the rhizosphere. Both NH_4^+ assimilation and Al tolerance depends on the same carbon-skeleton supply and is root-based, while NO_3^- assimilation is mainly foliar. Thus NH_4^+ nutrition and Al toxicity may be mutually antagonistic.

The hypotheses that were set out to test in this study were:

Hypothesis 1: The form of inorganic nitrogen (NO_3^- or NH_4^+) supplied will modify the responses of sugarcane to Al. NH_4^+ nutrition could exacerbate Al toxicity because NH_4^+ nutrition causes a reduction in rhizosphere pH and also requires carbon skeletons from the root for assimilation. This hypothesis was assessed by measuring the influences of NO_3^- and NH_4^+ with and without Al on the growth (dry matter accumulation, shoot:root ratios, RGR), Al, P, and N accumulation, N uptake, ^{14}C incorporation and the exudation of ^{14}C from the sugarcane varieties N12 and N19.

Hypothesis 2: The growth medium is likely to influence the response to the presence of Al. In liquid culture organic acids excreted by the roots to immoboilise Al would readily diffuse away from the roots reducing the effectiveness of these for detoxification of Al in the rhizosphere. In

4

contrast, in sand, organic acids are likely to remain more localized and therefore more effective for complexation with Al. This hypothesis was investigated by cultivation of plants in both liquid and sand culture with NO_3^- and NH_4^+ nutrition combined with Al. Both N12 and N19 sugarcane varieties were utilised.

1.2 Literature review

1.2.1 Al toxicity

The physiological basis of mechanisms of Al resistance and toxicity in plants continues to remain poorly understood, despite current interest in this field (Kidd *et al.*, 2001). Since 1978, a significant portion of research on plant metal toxicity has focused on the mechanisms of Al phytotoxicity and genetically based resistance to Al (Kochian, 1995). The intense research effort on Al toxicity reflects the agronomic and economic importance of this problem: Al toxicity is one of the primary factors limiting crop productivity on acid soils (Foy, 1984; Foy *et al.*, 1978). Thus Al toxicity on acid soils has been identified as a major constraint for the production of maize, sorghum, and rice in developing countries (De la Fuente-Martinez & Herrera-Estrella, 1999). Al toxicity is one of the primary growth limiting factors associated with crop plants such as maize, wheat, sorghum and rice in acid soils (Foy, 1988).

Acid soils diminish agricultural productivity in many regions of the world, including substantial areas of the United States. Of the land currently devoted to the production of crop species, approximately 12% is composed of acid soils (Buchanan *et al.*, 2000). Worldwide, 20% of maize acreage, 13% of rice acreage, and 5% of wheat acreage is grown on such soils (Buchanan *et al.*, 2000). Most maize, sorghum, and rice cultivars are being used are susceptible to toxic Al in the soil, and decreases in yield of up to 80% as a result of Al toxicity have been extensively reported in the literature (Sanchez & Salinas, 1981). In particular, maize and sorghum production is severely limited in tropical areas of Africa, Asia, and Latin America where over 45% of the total land area in countries such as Zaire, Zambia and Ivory coast are covered by acidic soils (De la Fuente-Martinez & Herrera-Estrella, 1999).

To better develop Al resistant crops via plant breeding and biotechnology, considerable research on the fundamental processes that confer Al resistance in plants is underway (Kochian, 1995). During the past few decades, research workers in agronomy, physiology and molecular biology have contributed to our understanding of the mechanism of Al toxicity and tolerance in plants. Although several review papers have been published, the mechanism of Al toxicity and tolerance are

6

complicated because of the complex chemical nature of Al and they have not yet been fully characterized (Matsumoto, 2000).

1.2.2 Forms of Al that are toxic

It is estimated that approximately 30 to 40% of arable land in the world is acid (Matsumoto, 2000). Al is the most abundant metal and the third most common element in the earth's crust (Kochian, 1995). At neutral or weakly acidic pH, Al exists in the form of insoluble aluminosilicate or oxide. However as the soil becomes more acid, Al is solubilized into a phytotoxic form (Rengel, 1996). Soluble Al can be classified into several different groups: free or mononuclear forms of Al, polynuclear Al, and Al as a low molecular-weight complex (Kochian, 1995). As the pH increases, octahedral hexahydrate $Al^{3+} \cdot 6H_2O$ changes to $Al(OH)^{2+} \cdot 5H_2O$ and $Al(OH)^{1+} \cdot 4H_2O$. At a neutral pH, insoluble gibbsite ($Al(OH)_3$) (pH 7) is formed and aluminate anion ($Al(OH)_4^-$)(pH 7.4) dominates at an alkaline pH (Kinraide, 1991). A very toxic polynuclear Al species, Al_{13} can also form when Al solutions are partially neutralized with a strong base, but its natural occurrence and contribution to soil toxicity are unknown (Delhaize & Ryan, 1995). Plant growth is adversely affected in acid soil, although a genotypical difference in Al toxicity and tolerance exists among plant species. Therefore the adverse effect of acid soil on plant growth is strongly related to the toxicity of Al ion solubilized (Matsumoto, 2000). Al toxicity was implicated as early as 1918 as a cause of the inhibition of root growth of barley and rye in acid soil (Hartwell & Pember, 1918).

1.2.2.1 *Expression of Al phytotoxicity*

Whether or not Al toxicity is viewed as a root or a shoot-related phenomenon, several possible mechanisms by which Al may disrupt cellular function have been proposed. These include (1) disruption of membrane structure and function; (2) inhibition of DNA synthesis and mitosis; (3) inhibition of cell elongation; and (4) disruption of mineral nutrition and metabolism, (Taylor, 1988a).

1.2.2.2 *Visual symptoms*

Symptoms of Al toxicity are not clearly diagnostic (Foy *et al.*, 1978). The first visual symptoms of Al toxicity are the reduction of root growth, and the toxic effects are evident in the root tips (Thawornwong & Van Diest, 1974). The most dramatic effects

are the reduction of both root and shoot growth. In a number of crops, symptoms resembling P deficiency, including small dark green leaves showing late maturation, purple coloration of stems, leaves and leaf veins and chlorosis and necrosis of leaf tips have been reported (Taylor, 1988a). This might be because Al induces P deficiency by binding to P. In other plants, Al toxicity appears as an induced Ca deficiency, manifest as curling or rolling of young leaves and collapse of growing points or petioles. Al-injured roots are characteristically stubby and brittle (Foy *et al.*, 1978). Al could displace Ca, thereby causing this problem. Roots tips and lateral roots become thickened and turn brown. The root system as a whole is coralloid in appearance, with many stubby lateral roots but lacking in fine branching (Clarkson, 1965). Such roots are inefficient in absorbing nutrients and water. In general, young seedlings are more susceptible to Al than older plants (Foy *et al.*, 1978).

1.2.3 Physiological effects

Much of the physiological research on the mechanism of Al toxicity has involved a single plants species or variety. In general, Al has been shown to interfere with; cell division in plant roots; P availability in soils and in or on plant roots; root respiration; activity of certain enzymes governing the deposition of polysaccharides in cell walls; cell wall rigidity (by cross-linking pectins) (Foy, 1974). Moreover, in some acidic soils plant growth is not only affected by Al toxicity but also by low availability of some essential elements such as P, Ca, Mg, and Fe, some of which form complexes with Al and consequently are not readily available for root uptake (Haug, 1984). At the cellular level, disorganisation of the plasma membrane is one of the first signs of Al stress (Bennet *et al.*, 1987a). Frequently cells of the root cap become vacuolated, show disruption of Golgi function and plastid development, alteration of nuclear structure, loss of cytoplasm, and disintegration. Al-affected epidermal, endodermal, and cortical cells rapidly autolyse and become swollen or disrupted (Bennet *et al.*, 1987b). Meristematic regions of the primary and lateral root become disorganised to the extent that the root cap and vascular elements cannot be distinguished. Sites of root hair eruption at the base of root hairs fail to heal property (Taylor, 1988a)

1.2.3.1 *Root apex is the site of Al toxicity*

Direct evidence has demonstrated that the root apex is the primary site of Al-induced root growth inhibition (Kochian, 1995). The root apex (root cap, meristem, and the

8

elongation zone) accumulates more Al and plays a major role in the Al perception mechanism. Indeed, only the apical 2 to 3 mm of maize and pea roots needs to be exposed to Al for growth to be inhibited (Delhaize & Ryan, 1995; Matsumoto et al., 1996). Bennet and co-workers (1987b) have suggested that the root caps play a major role in perception of Al injury. Based on anatomical studies of maize roots, Bennet & Breen (1991) and Bennet et al. (1987b) observed rapid changes in the ultrastructure of root cap cells, including Al-induced alterations in the secretory pathway and suggested that Al could indirectly inhibit root growth via an unknown signal transduction pathway involving the root cap, apical meristem, hormones, and other putative signals. However, the onset and extent of inhibition of root growth under Al stress were the same between intact and decapped maize roots (Ryan et al., 1993), which argues against a major role for the root cap in either Al toxicity or protection against Al toxicity (Kochian, 1995). Therefore, cell elongation in the elongation zone is thought to be the major target for the inhibition of root elongation by Al (Matsumoto, 2000). Sasaki et al. (1997) reported that root elongation in Al tolerant wheat (Atlas 66) was inhibited after 3 h when they were exposed to 50 μM $AlCl_3$ in 0.1 mM $CaCl_2$ (pH 4.5). However, Sivaguru & Horst (1998) found that the distal part of the transition zone of the root apex of maize, where cells undergo a preparatory phase for rapid elongation, is the primary target of Al.

Morphological changes in the root are often induced by Al toxicity after hours of Al treatment. The root apex becomes thick with cracks on the surface. This cracking might be caused by the outwardly expressed pressure of the cells at the second and third layer of the cortex. It is very important to understand how the cells in a specific zone (i.e., the elongating and/or transition zone of the root) accumulate a large amount of Al and their elongation is inhibited by structural alteration at an early stage of Al toxicity (Matsumoto, 2000). The implication of phytohormones in the Al induce toxicity syndrome in roots is a further point of interest, especially in relation to the root cap as a source of hormones and its role in Al resistance (Barcelo & Poschenrieder, 2002). After short-term exposure to Al, increased Al resistance has been found in cowpea varieties with an extended root cap. A first hypothesis that implicated a hormone signal for the transduction of the Al effect from the root cap to the elongation zone was discarded because plants with excised root caps were found as sensitive to Al as those with intact caps (Ryan et al., 1993). However, several

recent investigations support the view that Al-induced alterations of hormones levels in roots can play a role in early responses to Al (Barcelo & Poschenrieder, 2002). At present the information on Al-induced alterations of root hormones levels is far too fragmentary for establishing a general hypothesis that allows us to understand the implications of hormonal regulation.

1.2.3.2 *Disruption of membrane structure and function*

The plasma membrane is located at the boundary of root cells and is rich in phosphates as phospholipids. Thus, the plasma membrane is the first potential target for Al. Al evidently alters the plasma membrane structure and function (Matsumoto, 2000), and the interaction of Al and plasma membrane has been studied intensively.

1.2.3.3 *Disruption of mineral nutrition and metabolism*

Phosphate nutrition

In many plants Al tolerance appears to be closely associated with P-use- efficiency. In certain cultivars of wheat and tomato and inbred lines of maize, Al tolerance coincides with the ability to tolerate low P levels in nutrient solutions, either in the presence or absence of Al (Foy *et al.*, 1978). The symptoms of Al toxicity resemble those of P-deficiency and it has been shown that supply of P to growth substrates has a protective effect against Al injury (Taylor, 1988a). Not surprisingly, this led to the speculation that the toxic effects of Al may be attributed directly to an Al-induced P-deficiency (Taylor, 1988a). Clarkson (1966) reported a rapid increase in the P content of roots of *Hordeum vulgare* which had been pretreated with Al. That author hypothesized that inorganic P was precipitated with an Al-hydroxide complex at the root surface or in the apoplasm, and visualized a continuous adsorption-precipitation reaction which would reduce entry of P into the root and subsequent translocation to the shoot. Reduced concentrations of P in shoots of *Hordeum vulgare* treated with Al have also been observed, which has been explained on the basis of a reduced root system maintaining equal amounts of P translocation per unit weight (Taylor, 1988a). Clarkson (1966) was unable to find evidence of direct inhibitory effects of Al on P uptake into the symplasm, or its subsequent translocation. Increased ^{32}P uptake by root of *Picea rubens* treated with Al was accompanied by decreased translocation of P to foliage (Cumming *et al.*, 1992). Also, short-term uptake of ^{32}P into excised and intact roots of several legumes increased with pretreatment with Al, while the

efficiency of translocation to leaves decreased (Andrew & VandenBerg, 1973). However, in whole plants of *Oryza sativa*, uptake of P was depressed by pretreatment with Al. Inconsistencies in the literature may be due to the effect of varying ratios of N to P and varied sources of N supplied in the growth medium. Both affect P accumulation in roots and the transport of P to shoots of Al-stressed plants (Taylor, 1988a).

The importance of Al-induced P deficiency has been downplayed because induced-P deficiency cannot account for the rapid toxic effects on root elongation and because the toxic effects of Al are ameliorated in a nonspecific fashion by phosphate, Ca, Mg, K, or simply by ionic strength (Rhue, 1979). Nonetheless, induced P deficiency would appear to be an important part of the total Al toxicity syndrome. This is suggested because of populations of *Agrosis capillaris* differing in tolerance to acid, Al-toxic soils also showed differential acid phosphatase activity (McCain & Davies, 1984). The acid soil populations showed the highest acid phosphatase activities under conditions of low P. Interestingly, Al-induced repression of phosphatase activity did not vary between populations. While Al-induced P deficiency may not be a likely candidate for a direct Al stress injury, effects in P metabolism are prime candidates.

Studies have confirmed that Al directly affects cytoplasmic P metabolism (Hanson & Kamprath, 1979; Pfeffer *et al.*, 1986), but these results are not consistent amongst researchers. In roots of *Hordeum vulgare* pretreated with Al, phosphorylation of hexose sugars was inhibited by Al, possibly through inhibition of hexokinases. Increases in the amount of ATP and other nucleotide phosphates were detected, and the fact that the specific activities of ^{32}P in ATP were similar in Al-treated and control roots suggested that the rates of ATP synthesis were unaffected by Al stress (Clarkson, 1966). In roots of *Glycine max*, increased ATP and pyruvate levels were observed under conditions of Al stress, but this was interpreted to be the result of stimulation of ATP synthesis (Hanson & Kamprath, 1979). A decrease in the incorporation of ^{32}P into both nucleotides and hexose phosphates was observed in root of *Onobrychris sativa* (Rorison, 1965), while a decline in free nucleotides and an increase in hexose phosphates in Al-treated root of *Pisum sativum* has been reported (Klimashevskii *et al.*, 1970). Al forms stable complexes with commercial preparations of ATP (Karlik *et al.*, 1983), and Al as a contaminant in commercial preparations was

11

sufficient to inhibit the activity of yeast hexokinase (Womack & Colowick, 1979). This inhibition was reversed by addition of P or chelating agents. These results correlate well with a reported decreased ATPase activity in Al-stressed *Pisum sativum* roots (Klimashevskii *et al.*, 1970). However, an increase in ATPase activity was later reported in the same species (Klimashevskii & Bernatskaya, 1973). Conflicting results with regards to the effects of Al on acid phosphatase activity in *Pisum sativum* root have also been reported (Klimashevskii *et al.*, 1970).

Treatment of *Zea mays* roots with Al reduced concentrations of several nucleotides (ATP, UDPG) and reduced respiratory rates compared to controls. The toxic effects of Al were clearly manifest after treatment. Addition of glucose to the treatment solution nullified the toxic effects of Al suggesting that limited detoxification of Al in the cytosol was an energy-dependent process (Pfeffer *et al.*, 1986). Taylor (1988a) concluded that these results suggest that Al may affect P metabolism via effects on ATP hydrolysis, P esterification, or the hydrolysis of phosphoric acid monoesters, possibly leading to significant disruption of enzymatic processes in root exposed to Al. The direction and magnitude of these effects is not well established.

1.2.4 Mechanisms of Al resistance

Mechanisms of Al resistance can be classified into external exclusion mechanisms and internal tolerance mechanisms (Taylor, 1988a; Kochian, 1995) The main difference between these two types is the site of Al detoxification: apoplasm (external) or symplasm (internal), (Ma, 2000). The proposed mechanisms for exclusion include immobilization at the cell wall, selective permeability of the plasma membrane, plant-induced pH barrier in the rhizospere and exudation of chelating ligands. By contrast, internal tolerance mechanisms include chelation in the cytosol by organic acid, compartmentation in the vacuoles, Al-binding proteins and evolution of Al-tolerant enzymes (Kochian, 1995; Taylor, 1991). Recent evidence shows that organic acids play an important role in both the internal and external Al detoxification (Ma, 2000).

12

1.2.4.1 Exclusion mechanisms

1.2.4.1.1 The role of the cell wall in Al exclusion

It has been speculated that because of the fixed negative charges lining the water-filled pores within the cell wall, the root cell wall might be a site of Al binding and immobilization, which would prevent Al from associating with the PM on entering the symplasm. For this mechanism to be successful, cell wall material would constantly have to be synthesized at the site of Al toxicity, which would be the normal case at the root apex. On the other hand, it has been hypothesized that Al resistance should be favoured by low cell wall cation exchange capacity (CEC) which would result in the binding of smaller amounts of Al within the cell wall (Blamey et al., 1990; Mugwira & Elgawhary, 1979; Vose & Randall, 1962). Inherent in this model is the assumption that Al binding within the cell wall is the first step leading to Al toxicity, presumably via Al uptake into the cell (Kochian, 1995). However, the root cell wall CEC of the Al resistant genotype was higher than that of the sensitive cultivars (Allan et al., 1990). Observations indicated that root CEC plays only a minor role in Al resistance (Kennedy et al., 1986) and that root Al content greatly exceeded the root CEC (Wagatsuma & Ezoe, 1985). Thus, there is certainly no consensus among researchers that root CEC plays a role in Al exclusion (Matsumoto, 2000). Furthermore, Kochian (1995) had technical and conceptual reservations concerning this hypothesis. First, in all the studies where root CEC was measured, the entire root system and not the root apex was used. Secondly, in many studies root CEC was determined on intact roots after harsh treatments such as exposure of roots to HCl followed by immersion in plasmolyzing concentrations of KCl. Finally, there is no real conceptual basis for the assumption that Al binding within the cell wall is a prerequisite for Al uptake (Kochian, 1995).

1.2.4.1.2. Al Efflux across the plasma membrane

Based on the observation that in some Al resistant wheat cultivars inhibition of metabolism causes an increase in root Al content, some researchers have hypothesized the existence of an active Al efflux at the root cell PM (Taylor, 1991). Presumably this would be an Al translocating ATPase (Lindberg, 1990). Based on what is known about plant ion transporters, an Al-ATPase would be an unlikely candidate for a transport system (Kochian, 1995). First, the inwardly directed electrochemical gradient for Al across the PM would be so large that the energy released via ATP

hydrolysis would not be sufficient to drive the transport of Al out of the cell. Secondly, given the activity of Al in the cytoplasm is probably in the sub-nanomolar range, a transport protein with a Km for Al of approximately 10^{-10} M is unlikely to exist (Taylor, 1991). According to Pellet *et al.*, (1995) on what is known about Al stimulated release of organic acids as an Al exclusion mechanism in wheat, it is more likely that inhibition of metabolism causes a reduction in this process, with a concomitant rise in root Al levels.

1.2.4.1.3 Al exclusion via alterations in rhizosphere pH

Al resistance has been suggested to be due to a plant-induced pH increase in the rhizosphere (Taylor & Foy, 1985). Because the solubility of Al is strongly pH-dependent, maintenance of a high solution pH may have reduced the solubility and toxicity of Al. Blamey *et al.* (1993) found that an increase in the pH of dilute nutrient solutions from 4.5 to 4.6 causes a 26% decline in solubilized Al concentrations. There have been many reports of a general correlation between Al resistance and transient increases in solution pH. However, such differences in plant-induced pH cannot be a primary cause of different Al tolerance because of the lack of clear experimental evidence (Matsumoto, 2000). The hypothesis that an Al exclusion barrier could be created by plant-induced increases in rhizosphere pH has received much attention in the literature and conceptually is an attractive hypothesis (Taylor, 1991). However no convincing evidence has been presented in support of this model. All the studies published in support of this model have measured changes in bulk solution pH, which is influenced primarily by the mature root regions and not the root apex, which are the site of Al toxicity in wheat, maize and sorghum (Calba & Jaillard, 1997). Even if this major problem is ignored, Taylor (1991) pointed out that many reports in the literature contradict this hypothesis. More importantly, in a study where spatial aspects of root apical rhizosphere pH were measured with pH microelectrodes in wheat cultivars that are often used as the standards for Al resistance (Atlas 66) and sensitivity (Scout 66), Miyasaka *et al.* (1989) found no difference in the rhizophere pH at the root apex between the absence of Al or during the first several hours of Al exposure.

Recently, by using a vibrating microelectrode at a radial distance of 20 and 50 μm from the root surface of root tip of Al-tolerant Arabidopsis *alr-104*, Degenhardt *et al.* (1998) found a clear increase of pH. The Al-tolerant mutant *alr-104* alkalinized the rhizosphere in the presence of Al, but the wild type did not. They also found that Al exposure to *alt-104* induced a twofold increase in net H^+ influx localized to the root tip. The increased flux raised the root surface pH of *alr-104* by 0.15 pH units, suggesting that Al resistance in *alr-104* is mediated only by pH changes in the rhizophere. Thus it is likely that Al-resistant in at least some plants is related to exclusion through alteration of rhizosphere pH. However, this is unlikely to be a universal mechanism in plants that are resistant.

1.2.4.1.4 Rhizodeposition of mucilages

Mucilage and border cells have been implicated in Al resistance mechanisms (Horst *et al.*, 1982; Miyasaka & Hawes, 2001). An exact evaluation of the role of these rhizodepositions in Al resistance is complicated by the fact that their production is strongly influenced by substrate impedance and composition (Barcelo & Poschenfieder, 2002). In the tropical root legume binding of Al to the negatively charged root-tip mucilage, visualized by haematoxylin staining, seems to prevent Al uptake (Corrales, 2000). Higher mucilage production was observed in the Al resistant wheat cultivar Atlas 66 than in a sensitive cultivar (Puthota *et al.*, 1991). However, no consistent pattern of coincidence between differences in mucilage production, binding of Al to mucilage and Al resistance in wheat or maize could be established (Li *et al.*, 2000a; Wagatsuma *et al.*, 2001). In snapbean cultivars, higher Al resistance was related to better border cell viability and to higher mucilage production by the border cells of the Al resistant cultivar (Miyasaka & Hawes, 2001). These authors propose that an Al-induced mucilage layer surrounding each of several thousand cells encapsulating the root tip could provide a significant barrier to Al uptake into the roots.

1.2.4.1.4.1 *Exudation of organic acid anions*

Over a dozen Al-tolerant plant species are known to secrete organic acids from their roots in response to Al treatment (Ryan *et al*, 2001). Citrate, oxalate and malate are some of the commonly released organic acid anions that can form sufficiently strong complexes with Al to protect plant roots (Ma *et al.*, 2001). Malate is released from the

roots of Al-tolerant cultivars of wheat (Delhaize *et al.*, 1993), citrate from Al-tolerant cultivars of snapbean (Miyasaka *et al.*, 1991), maize (Pellet *et al.*, 1995), *Cassia tora* (Ma *et al.*, 1997) and soybean (Yang *et al.*, 2000) and oxalate for buckwheat (Ma *et al.*, 1997a) and *Colocasia esculenta* (Ma & Miyasaka, 1998). Some plant species, such as Al-tolerant triticale, rapeseed, oats, radish and rye, both malate and citrate are released (Li *et al.*, 2000b). In some of these species the increased secretion of organic acids by these plants is localized to the root apex and depends upon the presence of Al in the external solution (Delhaize *et al.*, 1993; Zheng *et al.*, 1998b). It is not possible for all the Al in soil to be detoxified by the root exudates and nor is it necessary. The root apex is particularly sensitive to Al (Ryan *et al.*, 1993), therefore only the cations that immediately surround the apical root cells needs to be detoxified. Secretion needs to continue as the root apex moves through the acid soil to replace the organic acids that diffuse away from the root or are broken down by microorganisms. Thus, organic acids forming a protective sheath around the root apex from the toxic Al cations (Ma, 2000).

Two patterns of organic acids secretion have been identified. In *Pattern I*, no discernible delay is observed between the addition of Al and the onset of organic acid release. For example, in wheat and buckwheat, the secretion of malate or oxalate, respectively, was detectable within 15 to 30 min after exposure to Al (Delhaize *et al.*, 1993; Zheng *et al.*, 1998b). In *Pattern II*, organic acid secretion is delayed for several hours after exposure to Al. For example, in *Cara tora*, maximal efflux of citrate occurs after 4 h exposure to Al (Ma *et al.*, 1997b) and in rye, citrate and malate efflux increased steadily during a 10 h period (Li *et al.*, 2000a). In maize, it now appears that Al might trigger both a rapid efflux of citrate as will as a second delayed release mechanism, which increases during a 48 h period (Pellet *et al.*, 1995). The rapidity of the *Pattern I* responses suggests that Al activates a pre-existing mechanism and that the induction of novel proteins is not required (Ma, 2000). In this case, Al might simply activate a transporter on the plasma membrane to initiate organic anion efflux. By contrast, the delay observed in *Pattern II*-type secretion might indicate that protein induction is required. These induced proteins could be involved in organic acid metabolism or in the transport of organic acid anions.

For organic acids to detoxify Al in the rhizosphere, they must be transported from the cytosol to the apoplasm. At the near-neutral pH of the cytoplasm, organic acids are almost entirely dissociated from their protons and exist as organic acid anions. Although many types or organic acids are found in root cells, only one ore two specific organic acid anions are secreted in response to Al treatment for any given species (Ma, 2000; Ma *et al.*, 1997b). This suggests that a specific transport system for the organic acid anions exist in the plasma membrane. In wheat and maize, this transport system has been identified as an anion channel (Zhang *et al.*, 2001; Ryan *et al.*, 1997; Pineros & Kochian, 2001). Patch-clamp studies on protoplasts prepared from wheat roots showed that Al activates an anion channel in the plasma membrane that is permeable to malate and chloride (Ryan *et al.*, 1997; Zheng *et al.*, 1998a). When this response was compared in a pair of near-isogenic wheat lines that differed in Al tolerance at a single genetic locus, Al was found to activate inward currents that were both larger and more frequent in protoplasts from the Al-tolerant genotypes than the Al-sensitive genotypes (Zhang *et al.*, 2001). In a similar study on Al-tolerant maize that secretes citrate in response to Al treatment, Al was also found to activate an anion channel on the plasma membrane (Pineros & Kochian, 2001). A second study on maize reported that the Al-activated anion channel is permeable to malate and citrate anions and occurs more frequently in an Al-tolerant genotype of maize than an Al-sensitive genotype (Kollmeier *et al.,* 2001). In addition, this study found that in the Al activated channels, activity is restricted to cells localized in a narrow zone within the root apex. It is not known how Al activates these anion channels but three possibilities have been proposed (Delhaize & Ryan, 1995): 1) Al interacts directly with the channel protein to trigger opening; 2) Al interacts with a specific receptor on the membrane surface or with the membrane itself to initiate a secondary-messenger cascade which then activates the channel; 3) Al enters the cytoplasm and activates the channel directly, or indirectly via secondary messengers

Recent evidence from maize has shown that Al is able to activate the channel in isolated patches of membrane, indicating that secondary messengers are either not required or are membrane-bound (Pineros & Kochian, 2001). It is also unclear what the differences are between the Al-tolerant and Al-sensitive genotypes that enable a large release of organic acid from the tolerant genotypes, but little or none from the sensitive genotypes. There might be differences in the numbers of channel proteins in

17

the membrane of each genotype, in their permeability to organic anions or in their activation by Al (Ma *et al.*, 2001). Until either the channel proteins or the genes encoding these channels have been isolated, physiological approaches are required to answer these questions. However, the important conclusion from these studies is that it is the anion channel that regulates the Al-activated secretion of organic acids from the roots (Ma *et al.*, 2001).

1.2.4.1.4.2 *Exudation of phenolic compounds*

Root exudation of phenolic compounds has been described by many authors (Marschner, 1995). However, the implication of phenolics in complex formation with Al has received much less consideration than organic acid anions. Phenolics can reverse the toxic effects of Al on hexokinase (Taylor, 1988b) and root elongation (Wagatsuma *et al.*, 2001). However, at equimolar concentrations they are less efficient than citrate in complexing Al (Ofei-Manu *et al.*, 2001). This is especially important for simple phenols like catechol at low pH, where H^+ efficiently competes with Al for binding sites in 1:1 complexes. Therefore phenolic sites in themselves are considered as not important for complexation of Al in acid environments. However, by a deprotonation reaction the phenolics in the presence of carboxylic groups from organic acids can strengthen the interaction between Al and organic acid anion ligand, increasing the effective stability constant for the Al-organic acid anion complex (Driscoll & Schechem, 1988).

It has also been argued that phenolics may favor Al binding by organic acid anions by inhibiting rhizsophere microorganisms that degrade organic acids. Recent investigations found Al-induced exudation of the flavonoid type phenolics, catechin and quercetin, at the root tips of an Al resistant maize variety (Kidd *et al.*, 2001). There was a coincidence between tip exudation of catechin and quercetin and amelioration of Al toxicity in Al sensitive maize. In the Al resistant maize variety (*Sikuani*) the Al-induced exudation of catechin reached rates above 100 nmol per tip h^{-1}, while the rate reported for citrate did not exceed 1 nmol per tip h-1 , a rate similar to that reported by other authors (Gaume *et al.*, 2000; Kollmeier *et al.*, 2001). Investigations on a larger number of species are required to assess if this exudation of flavonoid-type phenolics is a peculiarity of certain Al-resistant maize varieties or a

common property of a larger group of Al resistant species (Barcelo & Poschenrieder, 2002).

1.2.5 Internal tolerance mechanisms

1.2.5.1 Chelation in the cytosol by organic acid

It is well known that some highly tolerant species can accumulate high concentrations of Al in the above ground parts without showing symptoms of Al toxicity. Buckwheat leaves accumulate >400 mg Al kg^{-1} dry weight after only a short exposure to Al solution and as much as 15 000 mg Al kg^{-1} dry weight when grown on an acid soil. *Hydrangea* plants can accumulate high concentrations of Al, exceeding 3000 mg Al kg^{-1} dry weight in leaves over several months growth and the sepals of the species change from pink to blue with increasing Al concentration (Ma *et al.*, 1997b). The blue colour of *Hydrangea* sepals is caused by the formation of a complex between dephinidin 3-glucoside, Al and 3-caffeoylquinic acid, where Al is thought to play a role in stabilizing an interaction between the two organic compounds (Takeda *et al.*, 1985). About 80% of the total Al in *Hydrangea* leaves is present in a soluble form and the Al concentration in the cell sap can be as high as 13.7 mM (Ma *et al.*, 1997b). Recent studies have shown that a least two of three Al accumulator species detoxify internal Al by forming Al-organic acid complexes. Complexes of Al-citrate (1:1) in *Hydrangea* leaves (Ma *et al.*, 1997b) and Al-oxalate (1:3) in buckwheat (Ma *et al.*, 1998) have been identified by Al-nuclear magnetic resonance. The stability constant of Al-citrate (1:1) and Al-oxalate (1:3) are significantly greater than that of the Al-ATP complex. A current hypothesis is that this chelation effectively reduces the activity of Al in the cytosol, preventing the formation of complexes between Al and sensitive cellular components, such as ATP.

1.2.5.2 Compartmentation in the vacuole

Compartmentation within the cell is shared between several organelles, of which the vacuole and the mitochondria are most important. Organic acid concentrations in the cytosol are relatively stable whereas those in the vacuole can vary by one or two orders of magnitude in response to nutrient availability and metabolic activity (Ryan *et al.*, 2001). Tolerance to Al could be achieved if Al was sequestered in sites, which are insensitive to Al, such as the vacuole (Taylor, 1988b). Mathys (1977) proposed a mechanism for Zn tolerance in which malate binds Zn in the cytosol and acts as a

19

"transport vehicle" transferring Zn to the vacuole. Once in the vacuole, malate would be replaced by oxalate or another organic acid, which forms a stable chelate with Zn, and malate would be free to diffuse back into the cytosol. This tolerance mechanism has received some experimental support from nuclear magnetic resonance studies, which provided direct evidence for vacuolar compartmentation of Mn (Tayor, 1988b). Unfortunately application of this technology to the question of Al compartmentation in the vacuole presents some difficulties. While compartmentation in the vacuole has received support as a mechanism of tolerance to other metals (Taylor, 1987), evidence supporting compartmentation of Al is lacking. However, Clarkson, (1969) pointed out that in meristematic root cells, those most affected by Al treatment, are not vacuolated in either Al-tolerant or Al-sensitive species. Thus, in order to reduce damage to these tissues, internal detoxification would require a mechanism other than transport to the vacuole.

In *Melastoma*, Al was found as the free ion as well as in 1:1, 1:2 and 1:3 complexes with oxalate (Watanabe *et al.*, 1998). Except for the 1:3 complexes with oxalate, these forms of Al are potentially toxic to plants, therefore in *Melastoma*, it is possible that they are stored in the vacuole. Some species, for example buckwheat, also secrete organic acids, and it is unclear as to the relative contributions that internal and external organic acids make to the overall Al tolerance of the plant. In buckwheat, changes in the chemical form of Al occur during uptake, translocation and accumulation. The form of Al taken up by the roots is probably Al^{3+} because of the large inwardly directed electrochemical gradient for this ion, but the exact mechanism for uptake is unknown (Ma & Hiradate, 2000). Once taken up Al is chelated internally by oxalate to form a 1:3 complex (Al-oxalate) in the root cells (Ma & Miyasaka, 1998). When the Al-oxalate complex is loaded to the xylem, a ligand exchange reaction occurs to form Al-citrate (Ma & Hiradate, 2000). When Al is unloaded from the xylem to the leaf cells another ligand exchange reaction occurs to reform the Al-oxalate complex, which is then stored in the vacuole (Ma & Hiradate, 2000).

1.2.5.3 Al-binding proteins

A growing body of literature indicates that several plant species possess low molecular weight, metal binding proteins which are inducible by pretreatment with certain metals, show high absorbance at 254 nm, and show low absorbance at 280 nm

20

(Wagner & Trotter, 1982). These proteins share some of the characteristics of metallothionins isolated from animal tissues and could be involved in detoxification of metals in the cytosol (Taylor, 1988b). As in most other areas of Al resistance, little evidence exists in support of Al induction of this tolerance mechanism. The most widely cited work in this area is that of Aniol, (1984) who reported that preincubation of wheat seedlings with sub-lethal level of Al for 48 h conferred substantial Al resistance to both Al resistant and sensitive wheat cultivars. The same experiment was repeated with different Al resistant and sensitive wheat cultivars and no evidence was found for Al induction of Al tolerance (Rincon & Gonzales, 1991).

Several studies have showed that in the presence of moderate Al levels, root elongation declined initially followed by a partial recovery (Cumming *et al.*, 1992). These observations suggest induction of some type of Al resistance and warrant further study. However, mechanisms of Al resistance that are rapidly triggered by Al exposure, as in the case of organic acid release, could account for similar root growth responses to Al. Several laboratories have also shown that Al induces the synthesis of a number of different proteins which were found to be induced in both Al resistant and sensitive plants (Rincon & Gonzales, 1991). A few proteins may be induced or enhanced as part of a cultivar-specific mechanism of Al tolerance (Basu *et al.*, 1994). Taylor *et al.* (1997) found a 51-kDa protein induced in Al tolerant wheat (PT741) under Al stress. In contrast, this protein was not observed in an Al sensitive cultivar *Katepwa*. They suggested that the specific induction of the 51-kDa protein may play a potential role in mediating resistance to Al (Matsumoto, 2000).

1.2.5.4 The complexity of Al tolerance
Genetic variability of Al tolerance has been reported among a large number of important crop species (Lafever & Campbell, 1978). Wheat, perhaps the most comprehensively studied crop with respect to Al tolerance, serves as a useful model for investigating the genetic complexity of this characteristic (Carver & Ownby, 1995). Most studies of Al tolerance inheritance in wheat have found that this trait can be controlled by one or a few major genes (Dvorak, 1983). Compared to wheat, the genetic basis of Al tolerance in other crops has not received as much attention (Buchanan *et al.*, 2000). The genetic control of Al tolerance in barley has been attributed to the action of a single gene, with evidence that variation in the degree of

21

Al tolerance among cultivars is conditioned by different alleles of that gene (Reid & Anderson, 1996). Likewise, relatively simple inheritance of Al tolerance in maize has been reported, and as with barley, multiple alleles at a single locus also have been reported to confer various extents of Al tolerance to different inbred lines (Lindon *et al.*, 1997). In sorghum, Al tolerance has been reported as being either simple inherited or polygenic trait (Buchanan *et al.*, 2000). In rye Al-tolerance genes have been localized to chromosomes 3R, 4R and 6R by the use of wheat/rye chromosome addition and substitution lines (Buchanan *et al.*, 2000). Finally, although variability for Al tolerance has been reported in rice (*Oryza sativa*), no formal Al-tolerance inheritance studies in this species have been carried out (Khatiwada *et al.*, 1996). In relation to the global importance of grain crops, our understanding of the genetics of Al tolerance is limited, which is particularly surprising considering that large acreages of these crops are grown on acid soils (Buchanan *et al.*, 2000).

1.2.5.5 Al-tolerant enzymes

The active centres of many enzymes occupy a relative small portion of the molecule; large numbers of possible variation in the amino acid sequences of various enzymes could exist without loss of catalytic properties (Taylor, 1988b). Thus it seems conceivable that opportunity exists for the evolution of metal-tolerant enzymes. In a factorial design, Woolhouse, (1969) assayed the acid phosphatase activity of excised roots of an acid soil-tolerant (Al-tolerant), a Pb-tolerant, and a calcareous soil ecotype of *Agrostis tenuis* grown under conditions of Al, Pb, and Ca stress. In each combination of treatment and ecotype, acid phosphatase activity declined (Woolhouse, 1970). However, activity was least inhibited under conditions of Al stress in the Al-tolerant ecotype, under Pb stress in the Pb-tolerant ecotype, and under Ca stress in the calcareous ecotype. At first sight, these data seem to suggest the existence of tolerant enzymes, but closer evaluation indicates that this need not be the case. If other tolerance mechanisms for Al, Pb and Ca stress were operating in the excised roots, then it would be expected that enzyme activity would be least affected in cases where the tolerance of the plant matched the stress imposed. This possibility was supported in research with four enzymes extracted from a Zn-tolerant, a Cu-tolerant, a Zn-Cu-tolerant, and a Zn-Cu-sensitive ecotype of *Silene cucubalis* (Mathys, 1975). In contrast to previous studies, metals were supplied to enzyme preparations *in vitro* and to excised roots *in vivo*. When metal were supplied in vitro,

22

nitrate reductase, malate dehydrogenase, glucase-6-P dehydrogenase, and isocitrate dehydrogenase were inhibited by metal stress and there was no evidence for the evolution of tolerant enzymes in the tolerant ecotypes. When metals were supplied *in vivo*, nitrate reductase activity for Zn-tolerant plants was stimulated by Zn concentrations that were inhibitory to enzyme activity from sensitive plants (Taylor, 1988b). Similar effects were reported for malate dehydrogenase and isocitrate dehydrogenase. Thus, the existence of differential enzyme activity among ecotypes of *Silene cucubalis* was dependent on the presence of intact cells. Damage to cell structure was followed by a loss of resistance to Zn. At present, the literature does not support the occurrence of Al-tolerant enzymes. This may reflect a lack of research activity rather than a lack of Al-tolerant enzymes. Research has also indicated the occurrence of Ni-tolerant isozymes of acid phosphatase in *Anthoxanthus odoratum* Cox & Thurman, (1978) and *Festuca rubra* Johnston & Proctor, (1984). Clearly further research is warranted.

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Chapter 2

Comparative response to Al toxicity of N12 and N19 sugarcane plants grown hydroponically

2.1 Summary

o   The alleviation of aluminium (Al) toxicity in hydroponic medium was investigated for NO_3^- or NH_4^+-fed N12 and N19 sugarcane plants.

o   Hydroponically grown N12 and N19 plants were supplied with NO_3^- or NH_4^+ nutrition and treated with 0 mM or 1mM $Al_2(SO_4)_3$. Biomass characteristics, elemental concentrations of the whole plants, relative growth rate (RGR), N uptake rates and ^{14}C incorporation and exudation were measured.

o   In plants supplied with NH_4^+ nutrition, Al had no effect on the dry weights of N12 but increased that of N19 plants. Al treated NH_4^+-fed plants accumulated a small but significantly greater amount of Al and N compared to Al treated NO_3^--fed plants. The RGR in Al treated plants supplied with NH_4^+ nutrition was higher than that of plants supplied with NO_3^- nutrition. The uptake rate of NO_3^- by NO_3^--fed roots was generally higher compared to fraction in NO_3^--fed N12 plants was increased by Al compared to the N19 plants that the uptake rate of NH_4^+ by NH_4^+-fed roots. The amount of ^{14}C incorporated in the soluble fraction in NO_3^--fed N12 plants was increased by Al compared to that in the N19 plants which was decreased by Al. Al had no affect on ^{14}C exudation in N19 plants while in N12 plants reduced ^{14}C exudation was observed in Al stressed NO_3^- and NH_4^+-fed plants.

o   It was concluded that sugarcane was more tolerant to Al when treated with NH_4^+ nutrition than with NO_3^- nutrition. This may be partially due to sufficient supply of carbon skeletons from C_4 photosynthesis to deal with the simultaneous challenge of NH_4^+ assimilation and Al detoxification. The fact that growth was improved with NH_4^+ and Al may indicate that NH_4^+ and Al cations competed for access to the root tissue rather than Al being the cause of increased growth of N19 compared to N12 plants.

2.2 Introduction

The effects of Al on agricultural productivity are of concern within the South African sugar industry where severe soil acidification has resulted from intensive sugarcane mono-cropping (Schroeder *et al.*, 1994). Strategies used within the industry to alleviate the negative effects of soil acidity and Al phytotoxicity have met some success (Schumann *et al.*, 1999). However, they do not offer a sustainable solution for a number of agronomic, economic and environmental reasons. Although two ancestral species of modern sugarcane cultivars, namely *S. officinarum* and *S. spontaneum*, are reported to have different degrees of tolerance to Al, with the latter being the more susceptible (Drummond *et al.,* 2001), commercial genotypes (*Saccharum* spp hybrids) are generally regarded as tolerant of Al (Hetherington *et al.*, 1986). N12 and N19 are cultivars rated within the South African sugar industry as tolerant of Al (Schroeder *et al.*, 1994; Watt, 2003) and are widely grown on acid soils (Watt, 2003). A major factor limiting crop productivity worldwide is the presence of acid soils (Somers *et al.*, 1996). Acid soils are infertile primarily as a result of the highly toxic levels of Al which become available to plants when the rhizophere is at a low pH (4.0 to 5.0) (Wright 1989). When plants are subjected to Al stress, the primary effect is the inhibition of root tip growth with subsequent stunted root development (Wright 1989; Bennet & Breen, 1991). When root elongation is inhibited at micromolar concentrations, Al is accumulated at the epidermis and outer cortex cells of the roots tip in most plant species, including wheat (Delhaize *et al.*, 1993). Further penetration of Al to the stele seems to be slow, resulting in a high Al content in the root and low content in the shoot.

Inhibition of root elongation is a sensitive response of plants to Al supply (Horst *et al.*, 1983). The toxic effect of Al is dependent on the Al species (Alva *et al.*, 1986b; Cameron *et al.*, 1986) and the ionic composition (Blamey *et al.*, 1983) of the supply solution and on plant Al tolerance (Horst & Goppel, 1986). Comparisons of the effect of NH_4^+ and NO_3^--N supply to Al tolerance are complex due to the fact that not only N source but also ionic composition, pH of the solution, and growth rate of the control plants without Al may be modified. It is therefore not surprising that contrasting effects of N source on Al tolerance have been reported (McCain & Davies, 1983; Fleming, 1983; Rorison, 1985). Increasing ionic strength of the solution and

34

increasing concentrations of Ca^{2+} (Alva *et al.*, 1986a) and to a smaller degree Mg^{2+}, K^+ and Na^+ (Kinraide & Parker, 1987) alleviated Al toxicity. In addition, even in the absence of increased solution ionic strength, higher concentrations of these cations have been shown to alleviate Al toxicity. NH_4^+ could have the same effect, explaining the lower Al sensitivity of plants grown in the presence of NH_4^+-N.

NH_4^+ detoxification in the roots is dependent upon the availability of sufficient carbohydrate reserves to provide the necessary energy and carbon skeletons for its assimilation (Claussen & Lenz, 1995). With NH_4^+ compared to NO_3^- nutrition, the net carbon fixation in roots is up to 3-fold higher in rice and tomato (Ikeda *et al.*, 1992) and about 5-fold higher in maize (Cramer *et al.*, 1993). Nitrate uptake may be controlled by negative feedback by tissue NO_3^-, the level of N compounds in plant tissue (Lee; Rudges, 1986) and carbohydrate availability (Mengel & Viro, 1978; Arnozis *et al.*, 1988). The enhanced need of carbon for the assimilation of NH_4^+ was also demonstrated by Magalhaes *et al.*, (1992), who found that the supply of exogenous carbon sources such as oxo-glutarate to NH_4^+-fed roots improved their growth. Cultivar differences in NH_4^+ tolerance or sensitivity may therefore be related to differences in production, transport, and shoot-root partitioning of soluble carbohydrates that are required for the assimilation for NH_4^+ (Schortemeyer *et al.*, 1997).

NH_4^+-fed plants favoured incorporation of ^{14}C into amino acids while NO_3^--fed plants allocated relatively more ^{14}C into organic acids. The amino acid composition was also dependent on the type of nitrogen supplied, and asparagine was found to accumulate in NH_4^+-fed plants (Cramer *et al.*, 1993). It has also been shown that some amino acids could have an inhibitory effect on NO_3^- uptake rate (Doddema & Otten, 1979; Breteler & Arnozis, 1985; Rodgers & Barneix, 1992; Muller & Touraine, 1992), suggesting the involvement of these N reduced forms in the regulation of nitrate absorption. On the other hand NH_4^+ uptake has been reported to be mainly regulated by total N demand of the plant and the flux of mobile N-forms among different root and shoot compartments (Oji & Izawa, 1972; Lee & Rudges, 1986). Plants have evolved complex systems to absorb NH_4^+ and NO_3^- from the rhizosphere and assimilate them into organic forms (Taylor & Bloom, 1998).

The aim of this study was to investigate whether NO_3^- or NH_4^+ nutrition in hydroponic medium could alleviate the influence of Al on the growth of N12 and N19 sugarcane plants. Biomass characteristics, elemental concentrations of the whole plants, relative growth rate (RGR), uptake rate and ^{14}C incorporation, fractionation and exudation were measured. Since sufficient supply of carbon skeletons can alleviate Al phytotoxicity, it has been hypothesised that plants that assimilate NO_3^- in the shoots and NH_4^+ in the roots are better adapted to Al supply, because of a genotype variation due to a higher flux of carbons between the roots and shoots, fixation and sufficient supply of carbon skeletons to the roots.

Abbreviations: $Al_2(SO_4)_3$, aluminium sulfate; RGR, relative growth rate; ^{14}C, radio- labelled carbon; $NaNO_3$, sodium nitrate; Fe, iron; EDTA, ethylenediaminetetraacetic acid; ANOVA, analysis of variance; LSD, least significant difference; SLA, specific leaf area; SE, standard error; TCA cycle, tricarboxylic acid cycle; DW, dry weight

2.3 Materials and methods

2.3.1 *Growth conditions*

Bud-break was induced in setts (portions of mature sugarcane stalks bearing buds at each node) of N12 and N19 on a 1:1 mixture of vermiculite and compost for 30d. Young plantlets (4-wk-old) were transferred into aerated hydroponics after rinsing the roots in distilled H_2O. Two (morphologically and physiologically) distinct root systems are produced during sett establishment comprised of sett roots (fibrous and anchorage roots) and plant roots (fleshy and absorptive roots); these were not distinguished in this study. The base of the shoots of each plant was wrapped in foam rubber and inserted through plastic lids; the culm segment was left attached to the plant. Each tank contained eight plants supplied with 20l Long Ashton nutrient medium (Hewitt, 1966) modified to contain 2 mM $NaNO_3$ or 2 mM NH_4Cl as the N source and 0.09 mM Fe EDTA as the iron source (pH 4.5). The solutions were replaced once a week for 8-10 wks. The pH of the solution was monitored daily and was found to be relatively stable but, was corrected if it deviated by more than 0.1 pH units. Plants were grown in a temperature controlled (minimum 15°C, maximum

30°C) greenhouse at the University of Stellenbosch during summer (October to January).

For treatment with Al, the plants grown in Long Ashton nutrient solution were transferred to solution containing only 2mM $CaSO_4$ (pH 4.5) or 2mM $CaSO_4$ combined with 1mM $Al_2(SO_4)_3$ (pH 4.5) for 8 to10 weeks. The RGR was calculated using the natural logarithms of the fresh weight of each plant. Plants were removed from the containers the roots gently blotted dry and weighed within 2 min of removal on a weekly basis. Plants were harvested 8-10 wks after transplanting. Roots were blotted dry and the plants were separated into shoots and roots components before weighing. The leaf areas were measured using a leaf area meter (Li-Cor, Lincoln, NE, USA). The plant components were dried at 80°C for 48 h and then milled to pass through a 0.5m mesh screen. Of the milled material 1 g was ashed at 480 °C for 8 h and then suspended in 5ml 5 M HCl. The warm suspension was diluted to 50 ml and filtered through Whatman number 2 filter paper. The samples were analysed for Al, P and N by using an inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyser with suitable standards (Bemlab, De Beers Rd, Somerset West, South Africa).

2.3.2 Nitrogen uptake and ^{14}C labelling

Seedlings grown as described previously with either NO_3^- or NH_4^+ nutrition and with and without exposure to Al were transferred to 300 ml containers to allow determination of uptake by depletion from the nutrient solution (n = 5). The seedlings were pretreated overnight in a Long Ashton nutrient solution in 1 mM $NaNO_3$ or NH_4Cl. The seedlings were transferred to 300 ml solution containing 2 mM $CaSO_4$, 10 mM MES (pH 4.5), 1mM $NaNO_3$ or NH_4Cl and either 0 or 1 mM $Al_2(SO_4)_3$ for the uptake measurements. Sub-samples (1ml) were taken at regular intervals over a period of 10 h. The pH of the nutrient solutions was measured, the NO_3^- concentration determined according to Cataldo *et al.* (1975) and the NH_4^+ concentration by the indophenol blue reaction (Solorzano, 1969). After 10 h, 42 nmol $NaHCO_3$ containing 0.093 MBq $NaH^{14}CO_3$ was added to the nutrient solution for 1 h. The plants were removed from the nutrient solutions, the roots rinsed in two volumes of 2 mM $CaSO_4$, blotted dry, and the plants divided in to shoots and root components. The components

37

were weighed, quenched in liquid N_2 and stored at -18°C. Sub-samples of the nutrient solution (500 µl) were acidified with 500 µl of 0.3 M HCl, shaken for 12 h on a rotary shaker and then counted in a Beckman Instruments LS 5000 TD liquid scintillation counter (California, USA) with 2 ml Ready-gel (Beckman) scintillation fluid. A 10 ml sample of the nutrient solution was evaporated under an air-stream and made up to 1 ml. This was separated into amino acid, organic acid and neutral fraction using ion exchange resins prepared according to Atkins; Canvin, 1971. Samples were passed through 1 ml Dowex 50 W-X8 and Dowex 1 W-X8 (Sigma, St. Louis, MO, USA) columns in 1ml disposable syringes followed by 25 ml 50% (v/v) ethanol. The eluate was collected as the neutral fraction. The amino acid fraction was then eluted from the Dowex 50 W column with 10 ml 2M HCl, while the organic acid fraction was eluted from the Dowex 1 W column with 10 ml 6 M formic acid. These fractions were evaporated and made up to 1ml. A 0.5-ml aliquot of the neutral fraction was acidified with 50µl 0.3 M HCl and shaken for 12 h in a fume-hood. The neutral fraction and 0.5 ml of the amino and organic acid fractions were combined with 2ml Ready-gel scintillation fluid and quantified in a liquid scintillation counter.

The plant components were homogenized with a VirTis 6303 mill (VirTis Company, Gardiner, NY, USA) in 50 ml 80% (v/v) cold ethanol and stored at -18°C for 48 h and then extracted for 60 min at 45 °C. The samples were filtered through Whatman 1 filter paper and the filtrate made to volume (75 ml). The insoluble material left on the filter paper was dried at 80°C for 24h and then a sub-sample was mixed with 500 µl 0.3 M HCl and shaken for 12 h. Soluene-350 (Packard, Ill, USA) was then added (2 ml) and shaken for a further 12 h and then counted by liquid scintillation counting after the samples had been mixed with 4ml of scintillation fluid. Sub-samples (500 µl) of the soluble fraction were acidified with 50 µl 0.3 M HCl and allowed to stand in a fume-hood for 24 h before the addition of 2ml scintillation fluid and quantification by liquid scintillation counting. The remainder of the soluble fraction was then evaporated almost to dryness under an air stream and then evaporated almost to dryness under an air stream and then made up to 30 ml with water and separated into amino acids, organic acids and neutral fractions using Dowex 50 W-X8 ion exchange resins as described above. Aliquots (1 ml) of the fractions were combined with 2 ml of scintillation fluid and quantified by liquid scintillation counting.

2.3.3 Statistical analysis

Results were subjected to ANOVA to determine the significance of differences between the responses to the treatments using Statgraphics 7.0 (1993). Homogeneity of variances was determined using Bartlett's test and where variances were not homogenous the results were log-transformed for statistical analysis. ANOVA was followed by Fisher's project LSD test ($P < 0.05$) to determine the differences between the individual treatments.

2.4 Results

2.4.1 *Biomass characteristics*

N19 plants accumulated more dry weight and leaf area in the presence of Al than did N12 plants, but these differences were probably related to initial growth. NO_3^--fed N12 accumulated less dry weight when treated with Al, whereas NH_4^+-fed N12 plants were unaffected by Al (Table 1). This was possibly a result of the slightly higher roots dry weight accumulation in the presence of Al. Dry weights of N19 plants supplied with either NO_3^- or NH_4^+ nutrition were increased by Al. The leaf area of NH_4^+-fed N19 plants was increased when treated with Al. The SLA of N12 plants was generally increased when treated with Al and was higher in NO_3^- than in NH_4^+-fed plants. The SLAs of the N19 plants were significantly reduced by Al when combined with NO_3^- nutrition. Shoot:root ratios of the N12 plans were significantly decreased by Al supplied with NO_3^- nutrition. However, Al did not influence the shoot:root ratios of N19 plants. The changes in shoot:root ratio were mostly the consequences of changes in shoot biomass, with root biomass less influenced by the treatments.

Table 1 Biomass characteristics of NO_3^-- and NH_4^--fed plants treated with 0 or 1 mM $Al_2(SO_4)_3$.

	NO_3^-				NH_4^+			
	N12		N19		N12		N19	
	0 mM Al	1 mM Al	0 mM Al	1 mM Al	0 mM Al	1 mM Al	0 mM Al	1 mM Al
Dry weight (g)								
Shoots	1.58 ± 0.04 b	0.92 ± 0.04 a	3.49 ± 0.04 c	4.36 ± 0.04 f	2.68 ± 0.12 d	2.44 ± 0.04 c	5.51 ± 0.06 g	6.63 ± 0.04 h
Roots	0.39 ± 0.04 a	0.34 ± 0.04 a	0.98 ± 0.05 c	1.30 ± 0.04 d	0.75 ± 0.04 b	0.89 ± 0.04 c	1.65 ± 0.04 e	1.73 ± 0.04 e
Plant	1.98 ± 0.08 b	1.26 ± 0.08 a	4.45 ± 0.09 d	5.65 ± 0.07 e	3.42 ± 0.09 c	3.33 ± 0.08 c	7.16 ± 0.10 f	8.37 ± 0.08 g
Areas								
Leaf area (m^2)	0.86 ± 0.05 ab	0.65 ± 0.03 a	2.25 ± 0.06 c	2.30 ± 0.10 c	1.05 ± 0.02 b	1.13 ± 0.04 b	3.09 ± 0.23 d	3.39 ± 0.16 e
SLA (m^2 kg^{-1})	0.054 ± 0.002 c	0.070 ± 0.001 e	0.065 ± 0.001 d	0.053 ± 0.022 c	0.040 ± 0.002 a	0.046 ± 0.001 b	0.056 ± 0.004c	0.051± 0.002bc
Weight ratio								
Shoot : Root	4.21 ± 0.36 d	2.80 ± 0.23 ab	3.59 ± 0.13 c	3.37 ± 0.10 c	3.66 ± 0.30 cd	2.76 ± 0.07 a	3.33 ± 0.04 bc	3.38 ± 0.07 cd

Sugarcane N12 and N19 plants were grown in hydroponics and treated with an Al containing solutions for 3 days per week. Weight ratios express the ratio of shoot to root dry weight. Specific leaf area (SLA) is the ratio of leaf area to leaf dry weight. Letters indicating whether the treatments had a significant effect follow the mean ± SE ($N = 8$). Different letters indicate significant differences within each row at $P < 0.05$ as determined by ANOVA with *post hoc* LSD.

2.4.2 *Elemental concentrations*

Both N12 and N19 plants accumulated a significant amount of Al (Table 2). However Al accumulation by N12 and N19 plants supplied with NO_3^- nutrition was higher than in plants supplied NH_4^+ nutrition. The P concentration in the plant tissue was not significantly influenced by the different treatments or cultivars. NO_3^--fed N12 and N19 plants accumulated less N when treated with Al compared to NH_4^+-fed plants N accumulation was unaffected by the Al supply. However N accumulation in Al treated NH_4^+-fed plants was higher than in Al treated NO_3^--fed plants.

Table 2 Elemental concentrations in N19 and N12 sugarcane whole plants treated with 0 and 1 mM $Al_2(SO4)_3$ expressed as μmol per gram dry weight.

Characters	Elemental concentration (μmol g^{-1})			
	N12		N19	
	0 mM Al	1 mM Al	0 mM Al	1 mM Al
NO_3^-				
Aluminium	4.47 ± 0.69 a	35.5 ± 1.91 c	2.30 ± 0.13 a	56.1 ± 6.21 d
Phosphate	64.1 ± 45.9 a	61.9 ± 23.1 a	33.5 ± 8.08 a	48.1 ± 10.2 a
Nitrogen	1049 ± 7.37 e	808 ± 16.7 ab	954 ± 62.4 cd	756 ± 9.88 a
NH_4^+				
Aluminium	0.98 ± 0.08 a	17.1 ± 1.65 b	0.56 ± 0.05 a	12.5 ± 0.91 b
Phosphate	34.1 ± 3.35 a	59.3 ± 18.3 a	34.3 ± 5.49 a	28.6 ± 5.58 a
Nitrogen	1033 ± 32.5 de	1033 ± 51.6 de	845 ± 62.4 b	891 ± 16.1 bc

Values represent the mean ± SE (*N*=8). Different letters next to values indicate significant differences between treatments tested using analysis of variance (ANOVA) with *post-hoc* LSD.

2.4.3 *Relative growth rates*

With NO_3^- nutrition, Al decreased the RGR of N12 while with NH_4^+ nutrition Al increased the RGR's (Fig. 1). However Al did not significantly influence the RGR of N19 plants supplied with either NO_3^- or NH_4^+ nutrition.
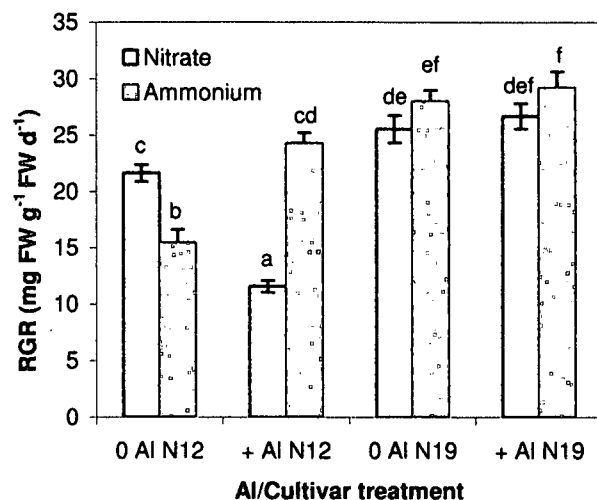
Fig. 1 The relative growth rate (RGR) (mg FW g^{-1} FW d^{-1}) of NO_3^-- and NH_4^+-fed plants treated with 0 or 1 mM $Al_2(SO_4)_3$. The RGR was calculated from the changes in fresh weight data measured during growth in hydroponics. Bars indicate the SE of means. The letters indicate whether the treatments had an significant effect ($P<0.05$, ANOVA with *post hoc* LSD, N=8).

2.4.4 *Nitrogen uptake rates*

The NO_3^- uptake rate of NO_3^--fed roots was generally higher than NH_4^+ uptake by NH_4^+-fed roots (Fig. 2). In the presence of Al uptake of NO_3^- by NO_3^--fed N12 and N19 roots was decreased. The uptake rate of NH_4^+ was not influenced by Al, but was lower in N19 than in N12 plants.

2.4.5 ^{14}C *incorporation and fractionation*

The amount of ^{14}C in the insoluble fraction was small relative to that in the soluble fraction (Fig. 3). The amount of ^{14}C incorporated into the soluble fraction of NO_3^--fed N12 shoots and roots were higher with Al, although Al decreased ^{14}C incorporation into the soluble fraction of NH_4^+-fed N12 shoots and roots. NO_3^--fed N19 shoots and roots incorporated less ^{14}C in the soluble fraction when supplied with of Al. However there were little changes in response to Al in N19 plants supplied with NH_4^+ nutrition soluble fraction.
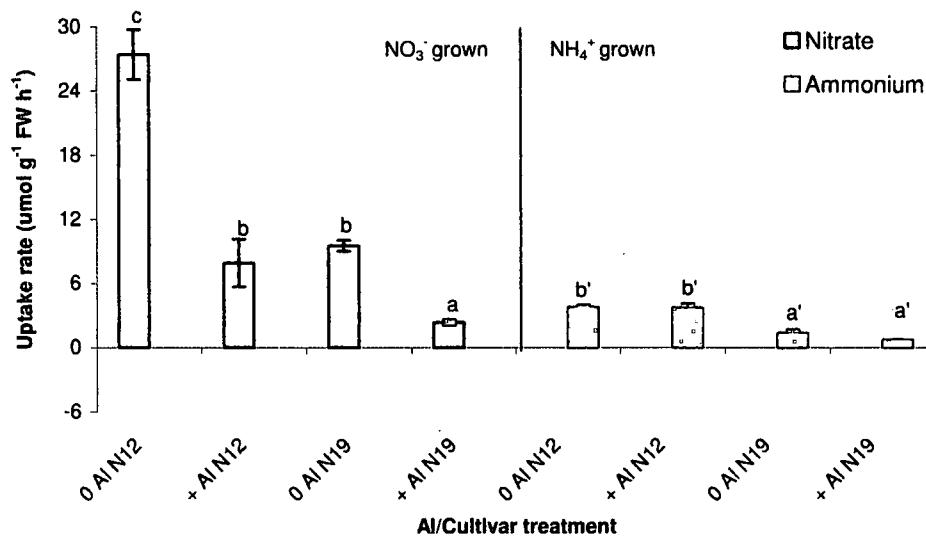
Fig. 2 The effect of NO_3^-- and NH_4^+-nutrition combined with either 0 or 1 mM $Al_2(SO_4)_3$ on NO_3^- and NH_4^+ uptake by N12 and N19 roots. Plants were grown with or without exposure to Al with either NO_3^- or NH_4^+ nutrition. During the uptake experiment (10 h) the plants were supplied with 1 mM $NaNO_3$ or NH_4Cl in a solution containing 2 mM $CaSO_4$ and additionally 1 mM $Al_2(SO_4)_3$ where indicated (pH 4.5). Bars indicate two SE of means. The letters indicate whether the treatment had a significant effect ($P<0.05$, ANOVA with *post hoc* LSD, $N=6$).

2.4.6 ^{14}C label allocation

The proportion of ^{14}C label allocated to the acidic fraction in the both N12 and N19 shoots and roots was not significantly influenced by the different N sources or Al treatments (Fig. 4). There were few and/or small significant differences in ^{14}C distribution in roots. Al did not significantly influence the amounts of ^{14}C allocated to the basic or neutral fractions in NO_3^--fed N12 roots and shoots. N19 plants supplied with NO_3^- allocated more ^{14}C into the basic and neutral fractions in the shoots than in the roots. With NH_4^+ nutrition, Al resulted in greater incorporation of ^{14}C into the basic fraction of N12 and a smaller incorporation with N19.
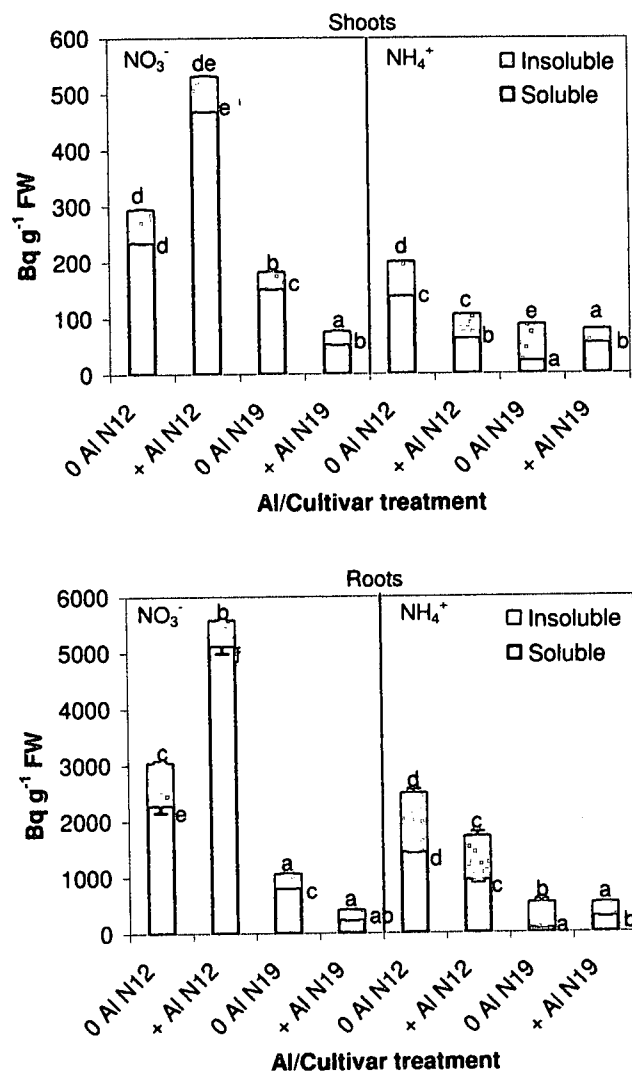
Fig. 3 Incorporation of ^{14}C supplied to roots for 1 h into acid-stable organic products (80% ethanol soluble and insoluble) in the shoots and roots of N12 and N19 sugarcane plants supplied with either NO_3^- or NH_4^+ nutrition. Plants were maintained in 2 mM $CaSO_4$ and (pH 4.5) with or without Al $(SO_4)_3$ for 10h prior to supplied ^{14}C. Bars indicate two SE of the means. The letters indicate whether the treatment had a significant effect ($P<0.05$, AVOVA with *post hoc* LSD, $N=6$).
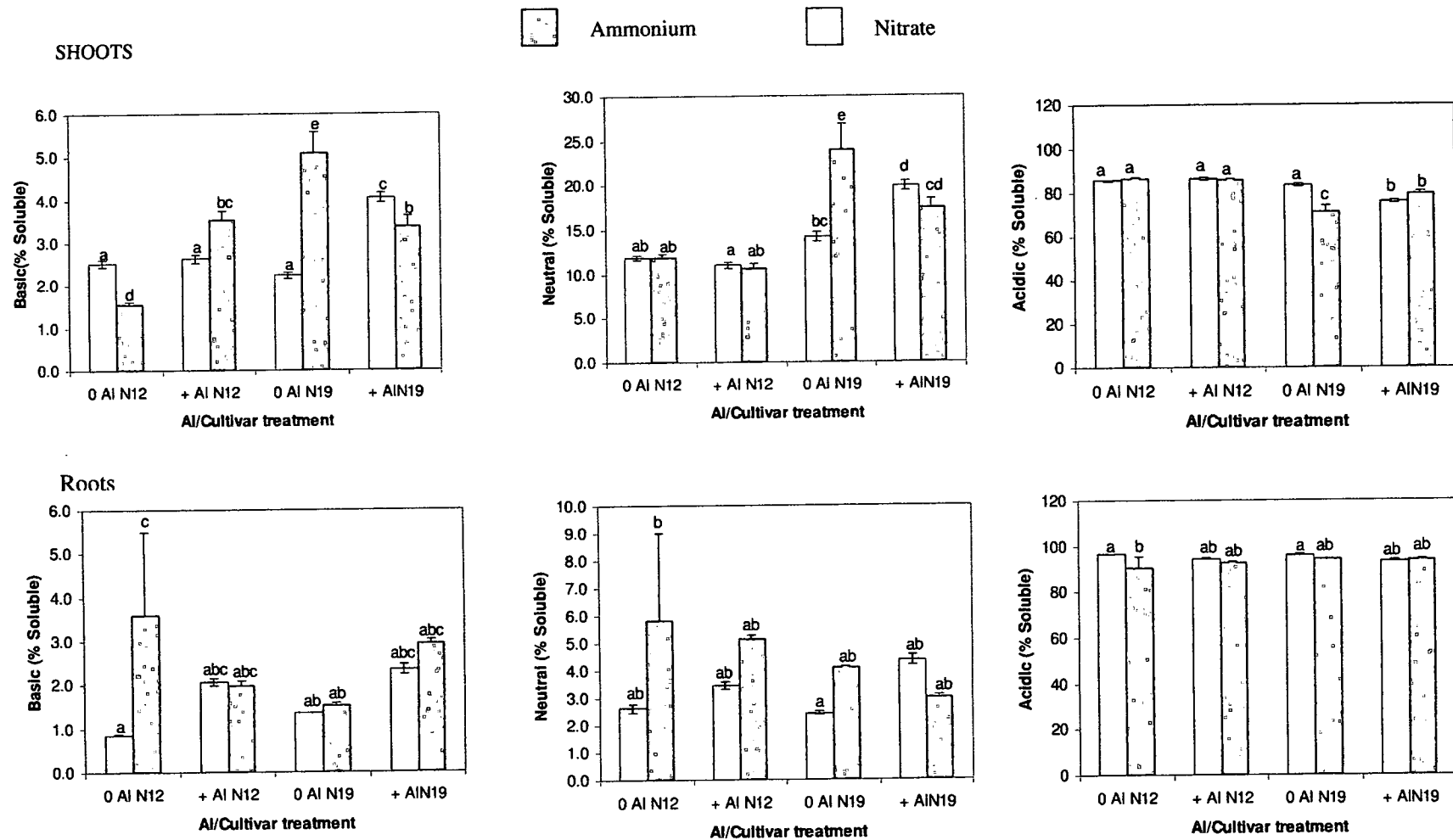
44

Fig 4. The proportion of ^{14}C (%) in the basic, neutral and acidic fraction in the shoots and roots of plants supplied with either NO_3^- or NH_4^+ nutrition. Plants were maintained in 2 mM $CaSO_4$ with or without 1 mM Al (pH 4.5) for 6 h before supply ^{14}C for 1h. Bars indicated the SE of the mean. The letters indicate whether the treatment had a significant effect ($P < 0.05$, ANOVA with *post hoc* LSD, $N = 6$).

45

2.4.7 ^{14}C label exudation

Exudation of acid-stable ^{14}C was significantly greater in NO_3^--fed N12 roots compared to NH_4^+-fed N12 roots in the absence of Al (Fig. 5). Exudation of acid-stable ^{14}C in N19 roots was very low and was unaffected by the N form or Al treatment.
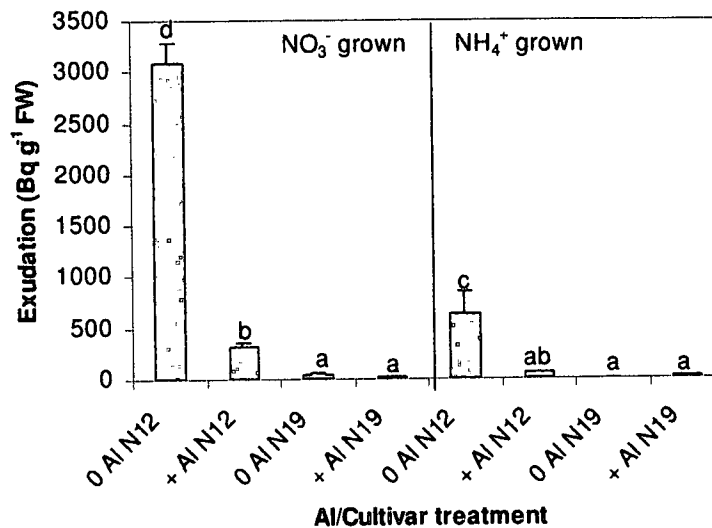

Fig. 5 The ^{14}C incorporated by roots and exuded by the roots during a 1 h pulse of ^{14}C. The 4 weeks old sugar cane N12 and N19 plants were grown with either NO_3^- or NH_4^+ nutrition. Plants were maintained in 2 mM $CaSO_4$ and (pH 4.5) with or without 1mM $Al_2(SO_4)_3$ for 10 h prior to supply ^{14}C. Bars indicate two SE of means. The letters indicate whether the treatment had a significant effect ($P<0.05$, ANOVA with *post hoc* LSD, $N=6$).

2.5 Discussion

The slow germination rate and slow initial growth of N12 plants compared to N19 plants (www.sugar.org.za\sasex\variety\index.html) are the consequences of a genotype variation between N12 and N19 cultivars and the cause of the lower dry weights of N12 compared to N19 plants (Table 1, Fig. 1). In the sugar industry N12 plants grown on 18 to 24 month cycle compared to N19 plants on 12 to 18 month cycle. The RGR was measured because the N12 and N19 had substantially different initial growth rates. Therefore, differences in overall dry matter accumulation are not particularly informative for between cultivar comparisons. N12 supplied with NO_3^- reacted adversely to Al, but when supplied with NH_4^+, Al stimulated growth. The lack

of response of the growth rates of the N19 cultivar to Al may indicate that the growth rates and carbon availability in this cultivar was high enough to enable tolerance to the supplied Al. These N19 plants did not exhibit strong variation in SLA or shoot:root ratios with Al indicating that Al did not alter carbon allocation within the plants. Since the Al was supplied in a relatively simple medium containing only $CaSO_4$ in addition to Al, most of this Al would be available to the plant in a soluble form (Watt, 2003). The fact that these plants were able to withstand 1 mM Al with little apparent influence on growth is quite remarkable. Watt (2003) found a high degree of tolerance in N19 sugarcane plants too, although, 1 mM was reported to have a suppressive effect on elongation of the tips of plant roots. The higher dry weights of NH_4^+-fed N12 compared to NO_3^--fed plants may be because roots of NH_4^+-fed plants form a stronger sink for carbohydrates than the roots of NO_3^--fed plants (Berta, 1976). This may lead to larger net carbon fluxes from the shoots to the roots for NH_4^+-fed plants (Lewis *et al.*, 1987). The greater tolerance of Al of the NH_4^+-fed N12 compared to NO_3^--fed N12 was probably due to increased growth of NH_4^+ compared NO_3^--fed plants. Al did reduce the shoot:root ratios of N12 plants with both NO_3^- and NH_4^+ nutrition indicating that Al accumulation did negatively influence shoot growth. This is a somewhat unusual response to Al because root growth is usually more strongly influenced than shoot growth (Kochian, 1995).

The smaller Al accumulation in NH_4^+-fed plants as compared to NO_3^--fed plants (Table 2) may be due to the protective effect of NH_4^+ nutrition on Al tolerance in the reported experiments could be explained by lower Al ad/absorption, as indicated by tissue Al concentrations and as previously reported for the roots of *Glycine max* (Klotz & Horst, 1988). These authors suggested that non-specific competition with positively charged Al species for binding sites within the apoplast was a likely explanation, rather than competition between NH_4^+ and Al for specific transport sites on the plasma membrane. The high effectiveness of NH_4^+ is probably due to its high affinity for binding sites, as was demonstrated for Ca (Alva *et al.*, 1986a; Horst, 1987). Thus the higher RGR of Al treated NH_4^+-fed plants (Fig. 1) could be explained by competition of Al and NH_4^+ for absorption sites in the apoplast (Antunes & Nunes, 1997). Al might therefore limit NH_4^+ uptake and conversely, NH_4^+ limit Al uptake. However, the rate of NH_4^+ uptake by the plants was not significantly altered by Al. NH_4^+ could inhibit movement of Al to or in the apoplast, preventing its interaction

47

with cell wall and plasma membrane constituents involved in cell elongation (Haug, 1984). Al could also possibly limit some negative impact of NH_4^+ on the growth of sugarcane.

It is well known that Al can inhibit NO_3^- uptake by interfering with the activities of the NO_3^- transporters (Durieux et al., 1993), and this could explain the lower N accumulation with Al in NO_3^--fed N12 and N19 plants. Durieux et al. (1993) also reported, upon removal of Al, a rapid increase of NO_3^- uptake in maize roots. Inhibition of NO_3^- uptake is clearly evident from the N uptake results (Fig. 2). The higher tissue N concentration with NH_4^+ than with NO_3^- in Al treated N12 and N19 plants indicates greater uptake of N from NH_4^+ source than NO_3^- in the presence of Al. This accumulation of N with NH_4^+ nutrition was not evident from the NH_4^+ uptake data. This is probably because, when exposed to NH_4^+, plants readily take it up and become saturated with N. Our experimental protocol did not allow for depletion of this accumulated tissue N prior to the uptake experiment. Thus the low rates of NH_4^+ uptake are probably attributable to the prior loading of N and tissue N-sufficiency.

The higher ^{14}C incorporation into the soluble fraction than into the insoluble in both N12 and N19 cultivars of shoot and root tissues (Fig. 3) probably reflects the relatively short time for the chase period (24 h) in this experiment. There were strongly contrasting patterns of ^{14}C allocation between cultivars and between plants treated with NO_3^- versus NH_4^+ nutrition. The largest incorporation of ^{14}C was in N12 supplied with NO_3^- and the strongest difference was between the N12 plants supplied with NO_3^- with and without Al. Al almost doubled ^{14}C incorporation in these plants. This Al-induced incorporation of root-zone inorganic carbon may have compromised growth in this treatment. Anaplerotic carbon fixation necessarily diverts carbon from the glycolytic sequence. Although this carbon may re-enter the TCA cycle, if it is diverted to other functions such as exudation of organic acids or amino acid synthesis, it could represent an important competitive drain of carbon from glycolysis-TCA metabolism, diminishing the functioning of other carbon-requiring processes (Cramer, 2002). With NH_4^+ nutrition, this N12 cultivar incorporated smaller amounts of ^{14}C, especially in the presence of Al. This was associated with increased growth of this

treatment in response to Al. Thus the protective interaction between NH_4^+ and Al might have reduced anaplerotic carbon fixation and reduced the costs of this to the carbon budget of the plant. In contrast to N12, N19 incorporated little ^{14}C, although Al significantly reduced the ^{14}C incorporation by this cultivar when combined with NO_3^- nutrition.

The differences in ^{14}C distribution between the acidic, basic and neutral fractions induced by the various treatments were small (Fig. 4). The source of neutral compounds may have been the shoot rather than the root, where these made up a greater proportion of the soluble fraction. These compounds may have resulted from photosynthetic refixation of respired $^{14}CO_2$ or direct decarboxylation of root-derived organic acids (Cramer, 2002). Most of the ^{14}C was apparently incorporated into the acidic fraction and this did not vary strongly between the various N and Al treatments. The greater exudation of acid-stable ^{14}C from NO_3^--fed N12 roots compared to NH_4^+-fed N12 roots (Fig. 5) could be because NO_3^--fed plants fixed more carbon skeletons into organic acids. ^{14}C exudation in N12 roots was inhibited by Al, probably because of the high levels of Al supplied in these experiments resulted in complexing of organic acids within the tissue thus decreasing available organic acids for exudation.

2.6 Conclusion

It was concluded that the effects of Al on plant growth could be partially ameliorated by the adequate supply of carbon skeletons from the C_4 photosynthetic pathways of sugarcane. The effect of the carbon skeletons is dependent on the N form and the consequent availability of carbon skeletons to the roots to alleviate Al toxicity. Thus NH_4^+-fed sugarcane plants were more Al tolerant than NO_3^--fed plants.

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Chapter 3

A comparison of the responses of N12 and N19 cultivars of sugarcane to Al toxicity when cultivated in sand

3.1 Summary

o   The potential amelioration of Al toxicity in plants grown in sand was investigated in either NO_3^- or NH_4^+-fed N12 and N19 sugar cane plants.

o   Sand grown N12 and N19 plants were supplied with either NO_3^- or NH_4^+ nutrition and treated with 0 or 1mM $Al_2(SO_4)_3^-$. Dry weight accumulation, shoot:root ratios and tissue concentrations of Al and P and N were measured.

o   Al had no effect on the dry weights of N12 or N19 plants supplied with either N source, but higher dry weight accumulation was observed in N19 plants supplied with NH_4^+. The shoot:root ratios of N12 and N19 plants were not influenced by Al, but N12 plants had higher shoot:root ratios. Both N12 and N19 plants accumulated more Al when grown in NH_4^+ nutrition. Higher P and N accumulated in N19 than in N12 plants supplied with NH_4^+ in the presence of Al.

o   It was evident from the results that the increased growth of Al-treated NH_4^+-fed plants might be due to mutual alleviation of both NH_4^+ and Al toxicity, by increasing root activity, organic acid excretion and stimulation of nutrient uptake by the roots.

3.2 Introduction

Aluminium is one of the most abundant metals and the third most abundant element in the earth's crust, comprising approximately 7% of the earth's crust (Buchanan *et al.*, 2000). Plant roots are therefore almost always exposed to Al in some form. The primary symptom of Al toxicity is a rapid (within minutes) inhibition of root growth, resulting in a reduced and damaged root system and limited water and mineral nutrient uptake (Barcelo & Poschenrieder, 2002). The rapidity of this response indicates that Al first inhibits root cell expansion and elongation; however, over the longer term, cell division is also inhibited (Matsumoto *et al.*, 2001). Because Al is so reactive, there are many potential sites including the cell wall, the plasma membrane surface, the cytoskeleton, and the nucleus that could be targets for injury (Kochian *et al.*, 2004). At neutral or weakly acidic pH, Al exists in the form of insoluble

aluminosilicate or oxide. However as the soil becomes more acid, Al is solubilized into a phytotoxic form (Rengel, 1996). $Al(H_2O)6^{3+}$, which by convention is usually called Al, is dominant in acid soil below pH 4.5 and is believed to be the most toxic form (Delhaize & Ryan, 1995). Many trivalent cations are toxic to plants and because Al toxicity is restricted to acid conditions, it is generally assumed that Al is the major phytotoxic species of this metal (Kinraide, 1991; Delhaize & Ryan, 1995).

Some plants species have evolved mechanisms to tolerate or resist Al stress, which help them to grow in acid soils (Ma *et al.*, 2001). Al resistance can be achieved through external mechanisms, which minimise uptake to Al, or internal mechanisms, which detoxify Al within the symplasm (Lazof *et al.*, 1997; Regel & Reid, 1997; Taylor *et al.*, 2000). Two external Al resistance mechanisms based on Al detoxification in the apoplast and rhizosphere have gained experimental support. The formation of non-toxic Al chelates with Al ligands secreted by root apices, and alkalinization of the apical apoplast and rhizophere, which shifts the concentration of mono-nucleur Al species in favour of less toxic Al hydroxides (Wenzl *et al.*, 2001). The effectiveness of Al resistant mechanisms based on external chelation of Al by chelating ligands may also be limited by other cell-surface ligands competing for Al ions (Parker & Pedler, 1998). Complexing of Al with P through the efflux of phosphate may be of questionable adaptive value in highly weathered acid soils where P may be the principle limiting nutrient (Wenzl *et al.*, 2001).

In addition to ameliorative effects of base cations and P, the plant available form of N is of importance, probably because the pH changes in the rhizosphere induced by NH_4^+ or NO_3^- uptake, with subsequent changes in Al solubility and speciation (Anderson, 1993). Plants tend to acidify the rhizosphere when NH_4^+ serves as the sole N-source and alkalinize the rhizosphere when NO_3^- serves as the sole N-source (Marschner, 1995). It has been claimed that increases in the pH of the soil solution brought about by plants may be due to preferential uptake of NO_3^- relative to NH_4^+(Antunes & Nunes, 1997). In contrast to NO_3^- uptake, uptake of NH_4^+ is associated with the release of excess H^+ associated with the NH_4^+ ion. The presence of Al in the rhizosphere can cause alterations in many physiological processes in the root including the uptake of NO_3^- (Rufty *et al.*, 1995). Thus there may be strong dynamic interactions between Al and N forms in the soil.

55

Camergo (1984) studied root elongation of nine cultivars of Brazilian wheat at different pHs of the nutrient solution combined with fixed Al concentrations. Camergo (1984) observed a positive relationship between the increase in pH and reduced plant sensitivity to Al. Klotz & Horst (1988) found that soybean root growth was less sensitive to Al in the nutrient solution combined with NH_4^+ than with NO_3^- as N source. The protective effect of NH_4^+ nutrition on Al tolerance could satisfactorily be explained by lower Al ad/absorption as indicated by Al concentrations of the root tips. However a non-specific competition with positively charged Al species for binding sites within the apoplast seems to be a more likely explanation than competition for specific transport sites within the plasma membrane (or plasmalemma). Antunes & Nunes, (1997) also concluded that, in the presence of toxic Al forms, the uptake of NO_3^- is more affected than the uptake of NH_4^+ and that the positive effect of NH_4^+ on root protection against Al is probably a more important factor than the strategy of pH increase. The satisfactory performance of plants grown in high external NH_4^+ concentrations is due to their ability to detoxify NH_4^+ via assimilation of NH_4^+ into amino acids in the roots (Givan, 1979). If NO_3^- is assimilated in the shoots, roots of NH_4^+-fed plants may form a stronger sink for carbohydrates than roots of NO_3^--fed plants (Berta, 1976). Thus protection offered by NH_4^+, as compared with NO_3^-, against Al toxicity should be offset by the costs of root assimilation of the NH_4^+.

Although many plant biologist prefer hydroponics as a growth medium for intensive agricultural research, when plants are grown hydroponically in the presence of Al, root exudates are readily lost from the rhizosphere compared to sand grown plants Also, when plants are grown in sand, organic acids excreted from the roots may bind to soil particles containing Al, resulting in reduced mobility and availability of the metal. Al toxicity has also been a major concern within the sugar industry. As Al tolerance is pH dependent, poor growth of plants on acid soils may reside from soil pH and not Al toxicity. The aim of this study was to investigate the influence of NO_3^- or NH_4^+ nutrition on Al toxicity in sand medium. Changes in dry weight accumulation shoot: root ratios, Al, P and N accumulation were measured in response to Al stress. The increased growth of Al-stressed NH_4^+-fed plants might be due to the alleviation of NH_4^+ and Al toxicity through organic acid excretion by the roots.

56

Abbreviations: $NaNO_3$, sodium nitrate; RGR, relative growth rate; EDTA, ethylenediaminetetraacetic acid; ANOVA, analysis of variance; LSD, least significant difference; SE, standard error; DW, dry weight

3.3 Materials and Methods

Bud break of lateral nodal buds on mature stalks of N12 and N19 was allowed to proceed in a 1:1 mixture of vermiculite and compost for 30 d. After 4 wks, plantlets were transferred into pure silica soil culture after rinsing the roots in distilled H_2O. Plantlets received Long Ashton nutrient medium (Hewitt, 1966) modified to contain 2mM $NaNO_3$ or 2 mM NH_4Cl as a N source (pH 4.5) for 2 weeks before treatment with Al. Plants were grown in a temperature controlled (minimum 15°C, maximum 30°C) greenhouse at the University of Stellenbosch during summer (September to January). For treatment with Al, plants received 2 mM $CaSO_4$ (pH 4.5) or 2 mM $CaSO_4$ combined with 1 mM $Al_2(SO_4)_3$ at (pH 4.5) for 3 consecutive days and Long Ashton nutrient medium for 4 days in a weekly cycle.

Plants were harvested 8 to10 weeks after transplanting. In order to harvest the plants, the pots were filled with H_2O and plants were carefully excavated. Roots were washed in distilled water and blotted dry and the plants were separated into shoot and root components before weighing. The plant components were dried at 80°C for 48 h and then milled to pass through a 0.5mm mesh screen. Of the milled material 1 g was ashed at 480 °C for 8 h and then suspended in 5ml 5 M HCl. The warm suspension was diluted to 50 ml and filtered through Whatman number 2 filter paper. The samples were analysed for Al, P and N by using an inductively coupled mass spectrometry (ICP-MS) and a LECO-nitrogen analyser with suitable standards (Bemlab, De Beers Rd, Somerset West, South Africa).

3.3.1 *Statistical analysis*

Results were subjected to ANOVA (Statgraphics 7.0, 1993) to determine the significance of differences between the responses to the treatments. Homogeneity of variances was determined using Bartlett's test and where variances were not homogenous, the results were log-transformed for statistical analysis. Where

percentage data was used it was arcsine transformed (Zar, 1984) before statistical analysis. ANOVA was followed by Fisher's projected LSD test (P<0.05) to determine the differences between the individual treatments.

3.4 Results

3.4.1 *Biomass accumulation*

3.4.1.1 *Dry weights*

Dry weight accumulation of sand grown plants supplied with either NO_3^- or NH_4^+ nutrition was greater for N19 than N12 plants (Fig. 1). Greater dry weight accumulation was observed in NH_4^+-fed N19 plants compared to NO_3^--fed N19 plants. Al had no effect on the dry weight accumulation of N12 or N19 plants supplied with either N source.
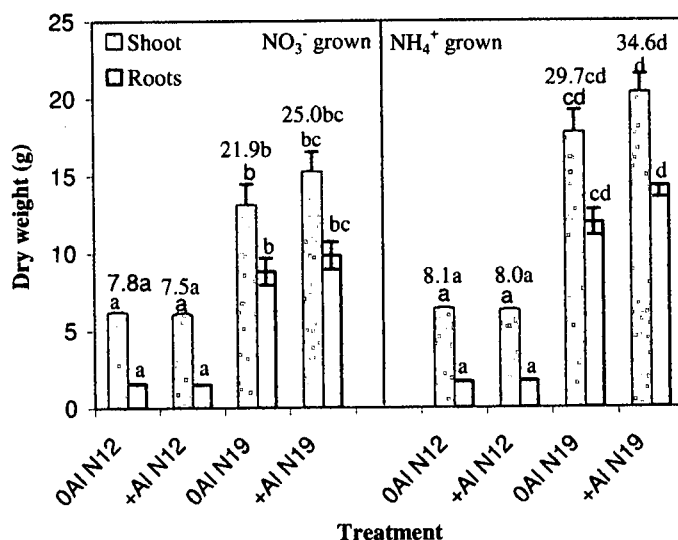

Fig. 1 The dry weight (g) of NO_3^- and NH_4^+ -fed N12 and N19 sugarcane plants treated with 0 or 1 mM Al_2 $(SO_4)_3$. The total plant dry weights are given above the bars. Bars indicate the SE of means. The letters indicate whether the treatments had a significant effect (*P*<0.05, AVOVA with *post doc* LSD, *N*=8). The shoots and roots were tested separately.

58

3.4.1.2 *Shoot: root ratios*

N12 plants had much higher shoot:root ratios than N19 plants (Fig. 2). Al had no influenced on the shoot:root ratios of N12 or N19 plants supplied with either N source.
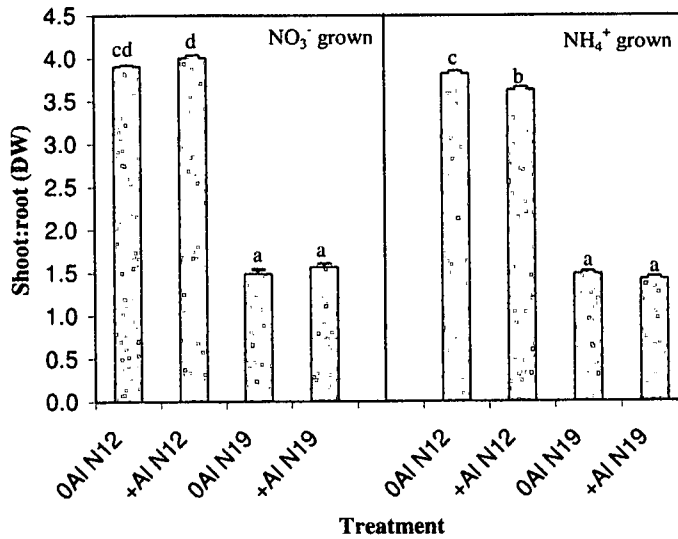

Fig. 2 The shoot:root ratios (DW) of NO_3^- or NH_4^+ -fed N12 and N19 sugarcane plants treated with 0 or 1mM $Al_2 (SO_4)_3$. Bars indicate the SE of means. The letters indicate whether the treatments effect ($P<0.05$, AVOVA with *post hoc* LSD,$N=8$)

3.4.2 *Tissue mineral accumulation*

3.4.2.1 *Tissue aluminium concentrations*

N19 plants accumulated greater concentrations of Al than did N12 plants grown with NH_4^+ nutrition (Fig. 3). Both N12 and N19 plants accumulated more Al in the roots than in the shoots. N19 accumulated more Al in the roots when grown with NH_4^+ compared to those grown with NO_3^- nutrition. No significant differences in accumulation of Al were observed in the roots and shoots of plants treated without Al and supplied with either NO_3^- or NH_4^+ nutrition.

3.4.2.2 *Tissue phosphate concentrations*

Plants treated with Al accumulated more P in both shoot and root tissue than did plants grown in the absence of Al (Fig. 4). P accumulation was more pronounced in the shoots than in the roots of both N12 and N19 plants. NH_4^+nutrition caused N19

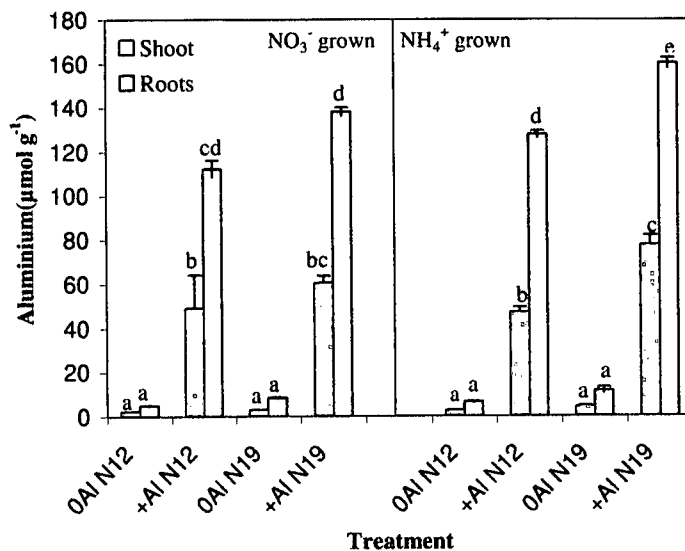and N12 plants to accumulate more P when supplied with Al than plants supplied with NO_3^- nutrition.

Fig. 3 The aluminium concentration (μmol g^{-1} DW) in the leaves of NO_3^- and NH_4^+ -fed N12 and N19 sugarcane plants treated with 0 or 1 mM $Al_2(SO_4)_3$. Bars indicate the SE of means. The letters indicate whether the treatments had a significant effect ($P<0.05$, AVOVA with *post hoc* LSD, $N=8$). The shoots and roots were treated separately.

Fig. 4 The phosphate concentration (μmol g^{-1} DW) in the leaves of NO_3^- and NH_4^+ -fed N12 and N19 sugarcane plants treated with 0 or 1 mM $Al_2(SO_4)_3$. Bars indicate the SE of means. The letters indicate whether the treatments had a significant effect ($P<0.05$, AVOVA with *post hoc* LSD, $N=8$). The shoots and roots were treated separately.
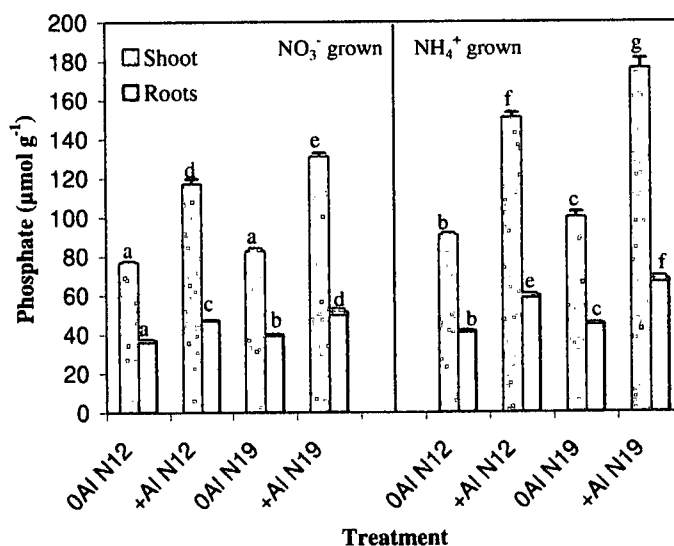
60

3.4.2.3 *Tissue nitrogen concentrations*

Plants accumulated more N in the shoots than in the roots (Fig. 5). NH_4^+-fed plants accumulated more N compared to NO_3^--fed plants regardless of the supply of Al. Although all plants exposed to Al had higher N concentrations, N19 plants accumulated even more N than N12 plants. Al less influenced nitrogen accumulation in the NO_3^--fed plants compare to the NH_4^+-fed plants.
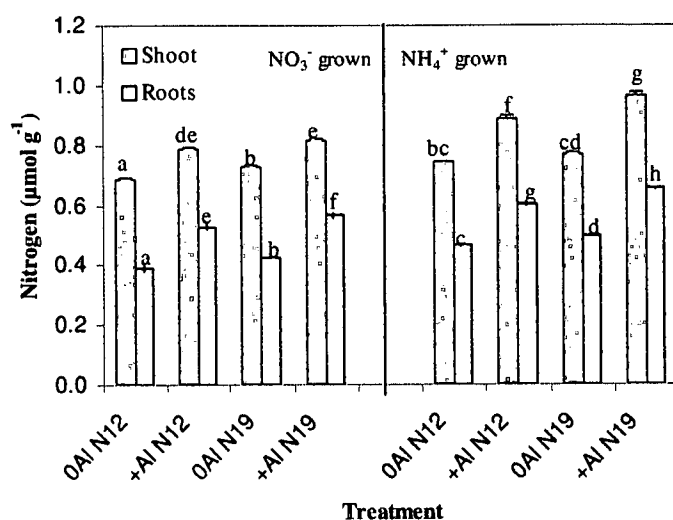

Fig. 5 The nitrogen concentration (μmol g^{-1} DW) in the leaves of NO_3^- and NH_4^+-fed N12 and N19 sugarcane plants treated with 0 or 1 mM $Al_2(SO_4)_3$. Bars indicate the SE of means. The letters indicate whether the treatments had a significant effect ($P<0.05$, AVOVA with *post hoc* LSD, N=8). The shoots and roots were treated separately.

3.5 Discussion

The higher dry weights of N19 plants compared to N12 plants (Fig.1) could be because N12 plants germinated slowly and early growth was generally slow while, N19 cultivar is best suited to early season growth (www.sugar.org.za\sasex\ variety\index.html). Due to this slow growth, N12 is grown on an 18-month cycle, rather than the annual cycle commonly used for other varieties, including N19. The higher dry weights of NH_4^+-fed N19 plants compared to plants grown in the absence of Al could be attributed to the competition of Al and NH_4^+ ions for absorption sites in the apoplast (Watanabe *et al.*, 2005). An enhanced tolerance of soybean root growth to Al when NH_4^+ was present has been reported (Klotz & Horst, 1988), and similar results have been observed in several sorghum genotypes (Tan *et al.*, 1992). The soybean results were interpreted to reflect an interaction in which NH_4^+ interfered

with binding of Al to membrane entities, thus offsetting the inhibitory influence of Al (Klotz & Horst, 1988). The higher dry weights of NH_4^+-fed N19 plants compared to NO_3^--fed N19 plants could be due to the ability of N19 plants to detoxify NH_4^+ via assimilation of NH_4^+ in the roots (Givan, 1979). Because the assimilation of NH_4^+ into amides and amino acids requires carbon skeletons for the tricarboxylic acid cycle (Oaks, 1992), roots of NH_4^+-fed plants may form a stronger sink for carbohydrates than roots of NO_3^--fed plants (Berta, 1976). However, C_4 maize has been shown to be able to tolerate NH_4^+ better than C_3 wheat (Cramer & Lewis, 1993). Thus sugarcane, which is a C_4 grass, may be able to tolerate high concentrations of NH_4^+ in the medium due to its capacity to assimilate the NH_4^+ into amino acids using abundant photosynthate. The lower dry weight of NO_3^--fed N19 plants compared to NH_4^+-fed N19 plants (Fig1) could possibly also be explained by lower tissue concentrations of N in the plants supplied with NO_3^- nutrition.

Al might have reduced tissue N in plants supplied with NO_3^- nutrition because of the influence of Al on the NO_3^- active transport mechanism, as was reported by Keltjens (1988) for sorghum and by Durieux *et al.*, (1993) for maize. The dry weights of N12 plants were unaffected by the form of N supplied (Fig.1), which indicates that these plants can shift their partitioning of carbohydrates towards the site where the bulk of N is assimilated or that biomass accumulation was constrained by other factors. The shoot is the predominant site of assimilation for N for NO_3^- plants and the roots are the site of N assimilation in NH_4^+-fed plants, as was demonstrated for maize plants by (Cramer & Lewis, 1993; Schortemayer *et al.,* 1997). Organic acids have been shown to play an important role in the tolerance of plants to Al either internally or in the root zone (Delhaize & Ryan, 1995; Ginting *et al.*, 1998; Ma *et al.*, 1998; Zheng *et al.*, 1998a; Zheng *et al.*, 1998b). The fact that N12 plants were unaffected by the Al treatment could possibly be due to the secretion of organic acids that chelate Al in the rhizosphere and render it non-toxic as was reported by (Delhaize & Ryan, 1995; Ma, 2000; Ryan *et al.*, 2001). In addition, the fact that Al had no effect on the growth of N12 plants could possibly mean that the plants were insensitive to the Al, resulting in the observed lack of response to the Al treatment.

The lower shoot:root ratios of N19 compared to N12 plants (Fig. 2) may be the result of the slower growth of the N12 than the N19 plants. The difference in shoot:root

ratio was mostly the consequence of smaller root extension in N12. The larger root of N19 may render these plants less sensitive to the deleterious effects of competition between root extension and root based NH_4^+ assimilation. The C_4 photosynthetic mechanism may be capable of providing sufficient carbohydrates to the roots to support NH_4^+ assimilation, Al detoxification and root extension. The fact that N12 plants had high shoot:root ratios may thus indicate that a large shoot biomass was required to support the functioning of the root in these plants. Thus the roots of N12 could possibly have an altered developmental or metabolic pattern requiring greater shoot activity.

Our observations indicated that N19 plants had longer roots and a larger root system which could have provided a greater area for Al accumulation, compared to N12 plants (Fig. 3). Both genotypes accumulated more Al in the roots than in the shoots. The root apex is extremely sensitive to Al, and attracts greater physical damage than the mature root tissue. Al can enter the symplast, probably as neutral Al ligands, by endocytosis, through membrane-bound proteins, or via stress-related lesions and be accumulated in the roots (Delhaize & Ryan, 1995). There was more Al in NH_4^+-fed N19 plants and more NH_4^+ taken up in Al treated N19 plants than in N12 plants. However, more Al accumulated with NH_4^+ nutrition than with NO_3^- nutrition and Al also resulted in an increased NH_4^+ uptake in both cultivars. Wenzl *et al.*, (2001) suggested that mechanisms based on physiological strategies, for example a low Al permeability of the plasma membrane, can also be responsible for the low Al accumulation in roots. Furthermore, Hue *et al.* (1986) reported the presence of organic substances, such as citrate and malate, may affect the availability of Al. When these complexes are exuded into the rhizosphere in the presence of Al, they can effectively chelate with Al and prevent its entry into the root (Pellet *et al.*, 1996), which may account for the lower Al levels that accumulated in the roots of N12 plants. Since plants supplied with NH_4^+ nutrition are known to divert carbon into amino acid synthesis at the expense of organic acid synthesis (Cramer & Lewis, 1993), it is possible than NH_4^+ nutrition increased Al accumulation in sugarcane due to reduced capacity for organic acid synthesis with NH_4^+. Such mechanisms of Al exclusion required carbon and may result in greater sink activity of the roots than in the shoots.

63

Higher P accumulation was observed in plants treated with Al compared to plants treated without Al (Fig. 4). The greater accumulation of P in plants exposed to Al is probably partially due to sequestration of P by Al due to the formation of Al-P complexes in the plant, rendering a portion of the P less available for metabolism. Furthermore, Wood & Cooper (1984, 1988) found that Al tolerance appeared to be closely related to the amount of P in the media or its availability in soil. Kirt et al., (1999 a,b) concluded that increased in P uptake by plants could be accounted for by the formation of soluble metal chelates rather than displacement of P ions from absorption sites or changes in rhizosphere pH.

Al-treated N12 and N19 plants accumulated more P when supplied with NH_4^+ nutrition than with NO_3^- nutrition, possibly because, as has been reported in many species, NH_4^+ increases P uptake. This might be a result of the fact that NH_4^+ is a cation and P is taken up as an anion. It has been well documented that, because of the high affinity of P for di-and tri-valent cations, citrate and other organic acids can displace P from insoluble complexes, making it more soluble and thus available for plant uptake Bradley & Sieling, (1953). The initial evidence for the role of organic acid root exudates in Al tolerance came from Kitagawa et al. (1986) who showed that differences in the Al tolerance of wheat genotypes were related to their capacity to exude malate. Thus Al-induced organic acid exudation might facilitate P uptake in these plants. Kitagawa et al. (1986) concluded that organic acid excretion confers a dual advantage to plants in acid soil, conferring tolerance to Al as well as enhancing insoluble Al-P use.

The higher N accumulation of NH_4^+-fed plants compared to those fed NO_3^- (Fig. 5) may be due to the more rapid uptake of NH_4^+ than of NO_3^- (Goyal & Huffaker, 1984). Plants exposed to Al and treated with either N source accumulated higher N than plants grown in the absence of Al. Growth stimulation by Al application was ascribed not only to the alleviation of acidity in the soil, but also to the increase of root activity for P uptake. It is generally recognized that Al exerts a toxic effect on plant growth and root activity. However, there have been several reports of the beneficial effects of Al on plant growth (Mullette, 1975; Konishi et al., 1985; Haridasan, 1988; Huang; Bachelard 1993). Kinraide (1988,1993) and Watanabe et al., (2005) showed that a stimulation of H^+ extrusion and an increase of transmembrane electrical potential

64

could be induced by 100 µM Al in wheat, which may account for the stimulation of nutrient uptake, and growth promotion. Malkanthi *et al.* (1995) reported that N and P absorption by *H. vulgare* roots were accelerated by Al, and that the Al-acceleration effects on P uptake disappeared when a metabolic inhibitor was used. Those findings indicated that the increase in P uptake was related to metabolic energy. Plants accumulated more N and P in the shoots than in the roots because N and P are essential nutrients and are acquired in larger quantities than other ions for photosynthesis (Lambers *et al.*, 1998). Investment of greater amounts of N and P in the shoots can increase photosynthetic capacity of the plant that in turn increased growth.

3.6 Conclusion

It was concluded that both N12 and N19 sugarcane plants are Al tolerant and that the high photosynthetic capacity of C_4 plants enable both cultivars tolerate Al, without a penalty on growth. Organic acid excretion could confer a dual advantage to the plants, by effectively chelating with Al and preventing its entry into the root, and by forming soluble complexes with nutrients, making nutrients more available for plant uptake. The increased growth of Al treated NH_4^+

-fed plants might be due to mutual alleviation of NH_4^+ and Al toxicity, by increasing root activity, organic acid excretion and stimulation of nutrient uptake.

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Chapter 4

4.1 General discussion and conclusion

Sugarcane (*Saccharum* spp hybrids) is generally regarded as tolerant of Al (Hetherington *et al.*, 1986). Nevertheless, despite this apparently high degree of Al tolerance, the extent and severity of soil acidification that arise under intensive monocropping (Schroeder *et al.,* 1994), suggest that even slight susceptibility to the metal may result in perceptible economic losses (Watt, 2003). Thus a better understanding of the physiological processes can be fundamental to the deployment of the Al toxicity management interventions that can offer a sustainable solution for a number of agronomic, economic and environmental concerns. Soil acidification has become a major problem in South Africa, and in KwaZulu-Natal it has been described as probably the most important factor limiting yield (Scotney & Dijkhuis, 1970), particularly in terms of the detrimental effects of toxic levels of exchangeable Al at low soil pH (Schroeder *et al.*, 1994). As a result much attention has been focussed on soil pH, and it has been recognized that acidity, rather than Al toxicity *per se,* may be the primary limiting factor in acid soils (Schroeder *et al.*, 1995). In our study we found that the effect of the different N sources may also be important in determining Al tolerance in sugarcane plants. When sugarcane plants were grown in acid soil at pH 4.5 and supplied with NO_3^- or NH_4^+ nutrition, NH_4^+-fed plants were less affected by Al as compared to NO_3^--fed plants. It is concluded that competitive interactions between NH_4^+ and Al might have partially excluded Al and limited the effects of this in plants supplied with NH_4^+ nutrition.

The sugarcane varieties N12 and N19 are probably quite Al tolerant due to the partial amelioration of Al by adequate supply of carbon skeletons from the C_4 photosynthetic pathways. The supply of sufficient carbon skeletons to the roots can enhance synthesis and exudation of organic acids. The ability of organic acids to chelate with Al and render it non-toxic is well established. It is well known that Al tolerant plants use organic acids, especially malate and citrate, to detoxify Al, both internally and externally (Delhaize & Ryan, 1995; Ryan *et al.*, 1995; Zheng *et al.*, 1998a; Zheng *et al.*, 1998b). However, the use of organic acids is likely to have an impact on the tricarboxylic acid (TCA) cycle and consequently the anaplerotic provision of carbon to the TCA cycle through the activity of PEPc (Cramer & Titus, 2001). Perturbation

70

of carbon flow through the TCA cycle due to the secretion of organic acids may result in less carbon being available for root growth. However, the C_4 photosynthetic pathway of sugarcane might make available a large supply of carbon skeletons to the roots for organic acid synthesis and Al detoxification without deleterious consequences for root growth.

As a result of the ability of sugarcane to detoxify NH_4^+ it is possible that roots of NH_4^+-fed plants may form a stronger sink for carbohydrates than roots of NO_3^--fed plants, if NO_3^- is assimilated in the shoots (Berta, 1976). Thus the sufficient supply of carbon might lead to larger net carbon fluxes from the shoot to the root for NH_4^+-fed plants. Several authors have found that the activity of PEPc in roots responded differentially to NO_3^- and NH_4^+ nutrition and that PEPc activity in the roots is increased by NH_4^+ compared to NO_3^- nutrition. Since both carbon availability and PEPc activity is dependent of the N source, the larger supply of carbon in NH_4^+ compared NO_3^--fed sugarcane roots might be lead to an increased amino acid synthesis through increase in PEPc activity. Since PEPc is also likely to be required for the anaplerotic provision of carbon for the synthesis of organic acids to detoxify Al, it seems likely that NH_4^+ nutrition would intensify Al toxicity. Since this was not the case in these experiments, it is concluded that the better growth of sugarcane with a combination of Al and NH_4^+ than with Al and NO_3^- may be due the afore-mentioned ionic exclusion due to competition between NH_4^+ and Al. Good evidence for exclusion of Al by NH_4^+ was obtained in hydroponically grown sugarcane, but not in sand-grown sugarcane.

It has been reported that Al can interfere with nutrient uptake (Rengel; Robinson, 1989; Haung *et al.*, 1992) such as P due the formation of Al-P complexes making P less available for plant uptake. However, sugarcane plants accumulated greater P concentrations when treated with Al and supplied with NH_4^+, compared to NO_3^- nutrition. Although the affinity of P for di-and tri-valent cations is high, citrate and other organic acids can displace P form the insoluble complexes, making it more soluble and available for plants uptake (Lopez-Bucio *et al.*, 2000). Greater availability of N with NH_4^+ than with NO_3^- nutrition in Al treated plants may promote plant development. This is likely because NH_4^+ is readily take up by plants resulting in plants becoming N saturated. Thus in sugarcane plants, growth stimulation by a

71

combination of Al and NH_4^+ may not only be due to competitive exclusion, but also due to the fact that NH_4^+ nutrition resulted in better root growth in hydroponically grown plants (Table 1). In this study there was some evidence that sugarcane sequesters more P and N in the shoots than in the roots (Fig. 4 and 5, Chapter 2), which could have increased the photosynthetic capacity of the plants and in turn increased growth.

The increased growth in Al treated NH_4^+-fed sugarcane plants, as compared to maize plants (Foy *et al.*, 1978) might be because sugarcane plants allocates sufficient carbon to organic acids and amino acid synthesis in the roots, as compared to maize plants that might allocate more carbon to organic acids or amino acid synthesis at the expense of root growth (Cramer & Lewis, 1993). According to these authors the lack of sensitivity of maize shoot:root ratios to NO_3^- and NH_4^+ nutrition may be a consequence of the low relative shoot:root ratios of maize with the consequence that the overall growth of maize is reduced in plants supplied with NH_4^+ nutrition, compared to plants supplied with NO_3^- nutrition. The importance of the shoot:root ratios are that they indicate the balance between photosynthetic and respiratory tissue in the plant. In the hydroponic experiment it was observed that changes in shoot:root ratios were mostly the consequences of changes in shoot biomass, with root biomass being less influenced by the treatments. Cramer & Lewis (1993) reported that the shoot:root ratios of maize grown hydroponically, in similar nutrition circumstances to the conditions used in the current study of sugarcane, resulted in a shoot:root ratios of close to 1. The fact that the shoot:root ratios of sugarcane were *ca.* 3-fold higher in sugarcane than in maize might indicate the reason for the different responses of sugarcane and maize to NH_4^+ and NO_3^- nutrition. This relatively high shoot:root ratio for a C_4 plant, which has high photosynthetic capacity, might partially explain the tolerance of sugarcane to Al in that sufficient supply of organic acids would be possible with high photosynthetic capacity.

Biologist prefer hydroponically grown plant material to sand grown plant material for research purposes for the reason that roots of soil grown plants are difficult to free of contamination from the soil and because the rhizosphere is less diverse (Miller & Cramer, 2004). This has resulted in hydroponically grown plants being use more intensive in agricultural research. Our results are in agreement with other reports (Foy

et al., 1978) that sugarcane plants, when grown in sand medium or hydroponically, prefer NH_4^+ over NO_3^- nutrition as a N source. It could be that the root apices of the hydoponically grown plants were exposed to higher Al levels than those of the sand grown plants. However, the plants grown in sand accumulated more Al than those grown in hydroponics. This is probably due to the differences in supply of Al between the two media, which cannot be matched exactly to one another. Thus there was no evidence for our hypothesis that organic acids can not form a protective "sheath" that shields the root apex for the toxic Al cations in the hydroponic solution because the organic acids simply diffuse away from the roots in a hydroponic system leaving hydroponically grown plants more susceptable to Al than sand grown plants. As in hydroponics, growth of sand-grown plants was enhanced by Al application, which could be associated with the excretion of organic acids from the roots. Secretion of organic acids continues as the root grows through the soil to replace the organic acids that may be lost by diffusion (Ma *et al.*, 2001). In the present study of sand-grown sugarcane, it was possible that organic acids could have to formed soluble complexes with cations, which made nutrients such as P more available for plant uptake and in return increased root activity. Evidence for this may be from the higher P levels in the tissue of Al-treated sand-grown, but not hydroponically grown plants.

The response of sand cultured plants to Al may be considered to be more of a "typical response" to Al, due to plants growing in an environment more similar to field grown plants which have probably evolved complex mechanisms to protect themselves from Al toxicity, compared to the artificial situation of hydroponically grown plants. It was therefore possible that Al may pose a greater problem to hydroponically grown roots than to sand grown roots. Negative charged soil particles may bind to Al and serve as a storage for Al and preventing its entry into the roots, making sand grown plants more tolerant to Al than hydroponically grown plants. In hydroponically grown plants the nutrient solution was continually replaced ever 6-7 days, and with the circulation of the solution Al could affect hydroponically grown plants more than sand grown plants. However, Al caused no change in the biomass accumulation of N12 and increased that of N19 in a rather uniform fashion in both sand and hydroponics. Although larger with NH_4^+, the biomass increase with Al was between 20 and 30% for both NO_3^- and NH_4^+ grown sugarcane plants. Thus no evidence was observed to

support a more negative responses of sugarcane to hydroponic growth with Al than in sand.

Future work should address which organic acids are secreted to provide a mechanism against the negative effects of Al and whether organic acids provide the main mechanisms of tolerance in sugarcane plants. More research is needed to investigate the allocation of carbon between organic acids and sugars in these high sugar-accumulating plants. Biotechnology may be a route through which the role of organic acids may be expanded, primarily the genetic modification of varieties to secrete greater levels of organic acids in response to Al challenge. In addition, the regulation of the organic acid pool (synthesis versus breakdown) should be further examined.

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74

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