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Aluminium tolerance in Brachiaria decumbens and Brachiaria ruzizenis

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Aluminium Tolerance in Brachiaria decumbens and Brachiaria ruziziensis

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Abstract

Aluminium phytotoxicity is becoming an increasingly widespread problem around the world, particularly in the tropics, causing decreased plant growth and crop productivity (Foy 1984; Delhaize and Ryan, 1995).

This study looked at the effect of aluminium on hydroponically grown *Brachiaria decumbens* (signal grass) and *Brachiaria ruziziensis* (ruzi grass), two very aluminium tolerant sub tropical grass species widely grown on sub tropical acids as cattle pasture and fodder. The study also looked at *Triticum aestivum cv atlas*, a tolerant wheat cultivar, for comparison.

Measurements of root growth inhibition and callose induction in response to increasing aluminium treatment were used to determine relative aluminium tolerance of the three species. *Brachiaria decumbens* root growth was found to be unaffected by treatment with 200 μM aluminium, whereas in *Brachiaria ruziziensis* 200 μM aluminium treatment caused 40% root growth inhibition and in *Triticum aestivum* it caused 100% inhibition.

Examination of the effect of aluminium treatment on nutrient uptake in *Brachiaria decumbens* and *Brachiaria ruziziensis* found that the superior adaptation of *Brachiaria decumbens* to nutrient poor acid soil conditions compared to the closely related *Brachiaria ruziziensis* was probably due in part to a better nutrient uptake system, particularly iron, and lower optimum nutrient requirements. It is also probably due in part to better maintenance of nutrient uptake and root and shoot nutrient concentrations when exposed to aluminium.

Induction of increased organic acid accumulation and exudation from roots is widely accepted to be a major aluminium tolerance mechanism (Kochian, 1995). Organic acid accumulation was not triggered by phosphate starvation, although organic acid

tissue accumulation was increased in response to general nutrient deficiency in root tips of *Brachiaria decumbens* and *Brachiaria ruziziensis*.

Brachiaria decumbens and *Brachiaria ruziziensis* were found to employ two separate organic acid aluminium tolerance mechanisms. The first primarily involved a large induction of citrate in root tissue for internal detoxification and sequestering of aluminium. The second mechanism involved exudation of malate and oxalate for chelating and excluding aluminium from root tip cells.

The greater aluminium tolerance of *Brachiaria decumbens* and *Brachiaria ruziziensis* than *Tricium aestivum cv Atlas* was found to probably be due to higher organic tissue concentrations, particularly citrate, and exudation of relatively high concentrations of oxalate and malate. Organic acid accumulation in root tip tissues did not account for the difference in aluminium tolerance between *Brachiaria ruziziensis* and *Brachiaria decumbens*.

Recent studies have implicated the oxidative stress system in aluminium tolerance (Cakmak and Horst, 1991; Yamamoto *et al.* 1997; Ono *et al.*1995; Richards *et al,* 1998). However aluminium was found to cause no increase in either superoxide dismutase, peroxidase or catalase in either *Brachiaria* species. Aluminium also had no significant effect on ascorbate and dehydroascorbate concentration, indicating that the oxidative stress system was not involved in aluminium tolerance in either *Brachiaria decumbens* or *Brachiaria ruziziensis*.

Contents

List of Figures

List of Tables

Abbreviations

Bd	*Brachiaria decumbens*
Br	*Brachiaria ruziziensis*
Atlas	*Triticum aestivum cv atlas*
S.E.	Standard error
SOD	Superoxide dismutase
POD	Guaiacol peroxidase
CAT	Catalase

Chapter One: Introduction

1.1 Structure of this thesis

The thesis comprises seven chapters; an introductory chapter, a methods chapter, four research chapters and a final concluding chapter drawing together the different strands of the thesis. All research chapters begin with a literature review on the chapter subject. The first two research chapters look at the measurement of aluminium toxicity and its effects on plant health and nutrition. The second two research chapters investigate two potential aluminium defence strategies.

Chapter One:

Overview of the causes and problems associated with low pH soils including aluminium toxicity. An introduction to the adaptation and tolerance of *Brachiaria decumbens* and *B. ruziziensis* to low pH soils, agricultural importance and current knowledge on their physiology and environmental adaptations

Chapter Two:

Methods chapter.

Chapter Three:

Measurement of the relative aluminium tolerance of *B. decumbens* and *B. ruziziensis* in comparison to an aluminium sensitive wheat cultivar, using various physiological markers such as root elongation, biomass, root tip callose concentration and photosynthetic ability.

Chapter Four:

Effect of nutrient stress on aluminium tolerance, and effect of aluminium on nutrient uptake.

Chapter Five:

Exploring the most widely accepted aluminium defence mechanism, the induction of organic acid production and exudation in response to aluminium treatment.

Chapter Six:

Examines whether the high aluminium tolerance of *Brachiaria decumbens* and *B. ruziziensis* is due to an increase in capacity of the oxidative stress system. Looking particularly at the effect of aluminium on peroxidase and superoxide dismutase activities and induction of increased ascorbate production.

Chapter Seven:

Looks at the overall picture of aluminium tolerance and defence in *B. decumbens* and *B. ruziziensis.*

1.2 Introduction

Aluminium phytotoxicity is becoming an increasingly big problem around the world, causing decreased plant growth and crop yields amounting to billions of pounds per year in lost revenue and with even greater ramifications for land under subsistence farming in the developing world (Foy, 1984; Delhaize and Ryan, 1995). Aluminium is one of the most abundant elements on earth (Kochian and Jones, 1996), however most is bound up in stable non-toxic forms such as silicates and is therefore unavailable to plants (Delhaize and Ryan, 1995). The problem of aluminium toxicity is greatest in the tropics, where high rainfall, geology and some farming practices produce large areas of land with low pH soils (van Wambeke, 1976). Here the aluminium becomes mobilised, available and toxic to plants. Aluminium is toxic to plants in micromolar concentrations (Owenby and Popham, 1989). In recent years considerable time and resources have been put into looking at the problem of aluminium phytotoxicity, with the long-term aim of producing resistant crops able to grow in these adverse conditions (Rao *et al.*, 1993).

Despite the huge interest in aluminium toxicity and resistance, relatively little is yet known about the chain of events leading to aluminium toxicity and potential mechanisms of resistance and accumulation, much of which is still speculative (Kochian, 1995).

1.2.1 Acid soils

Acid soils are widespread throughout the humid and sub-humid tropics (Figure 1.1, van Wambeke, 1976), where the geological conditions and continued exposure to rain are ideal for acid soil formation. They cover 850 million hectares in America, 450 million hectares in tropical Africa and 210 million hectares in tropical Asia (Rao *et al.*, 1993).

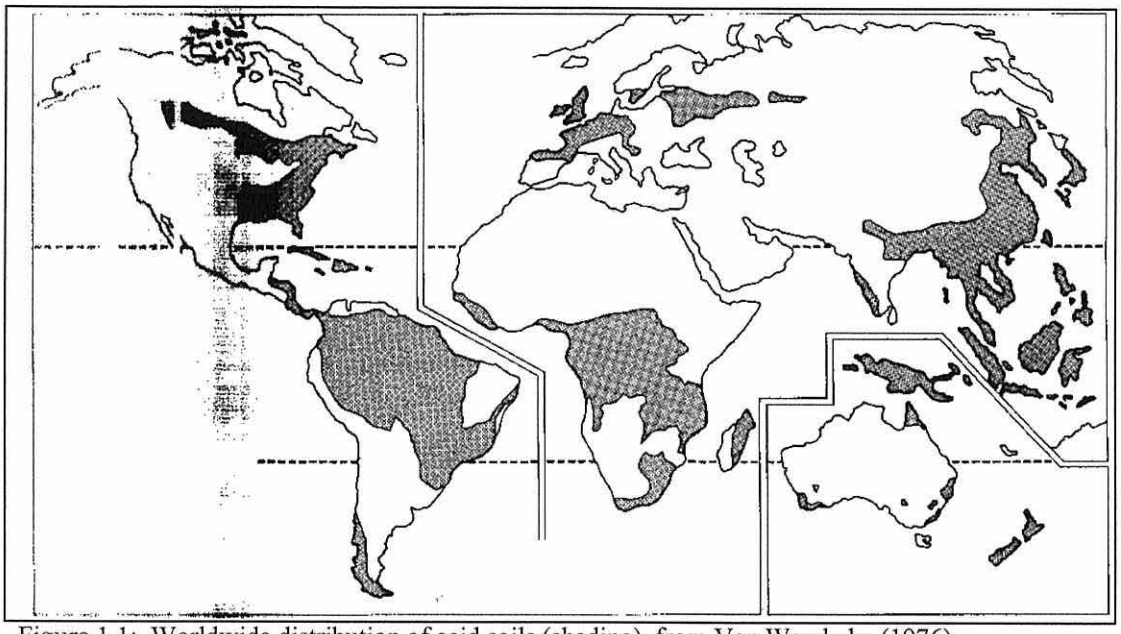

Figure 1.1: Worldwide distribution of acid soils (shading), from Van Wambeke (1976).

Acid soils are formed when nutrients and cations, such as magnesium, calcium and potassium, are leached out of the topsoil layers by rainfall (Bolan *et al.,* 1991; Blake *et al.,* 1999). The calcium, magnesium and potassium ions bound to the soil cation exchange sites are gradually replaced by protons, then aluminium and manganese ions, resulting in acidic soil conditions (Bolan *et al.,* 1991; Blake *et al.,* 1999). This natural process can be further exacerbated and the rate increased by anthropologic influences such as acid rain, and land management practices such as nitrogen fertiliser application (Kennedy, 1986).

The problems for plants growing on acid soils are various and complex and reviewed by Kamprath and Foy (1985). One of the major factors is the low fertility of acid soils (Foy, 1983). Leaching of nutrients such as nitrogen, phosphorus, calcium, magnesium, potassium, zinc, molybdenum and sulphur during soil acidification is a great limitation on plant growth (Foy, 1983). As well as the problem of leaching, the complex soil chemistry associated with acid soils can further reduce fertility. Aluminium mobilised in the low pH conditions binds phosphate, forming aluminium phosphate, which precipitates and becomes unavailable to plants (Clarkson, 1967). So even in acid soils with high levels of phosphate, maybe due to addition of

phosphate fertilisers, most is locked away as aluminium phosphate and unavailable for uptake by plants.

In addition to the low fertility status of acid soils, there is also a problem of toxicity caused by mobilised metal ions such as aluminium and manganese. Aluminium only usually becomes a problem when mobilised by pH levels below 5.0 (Kochian and Jones, 1996; Delhaize and Ryan, 1995), although aluminium toxicity has been shown in kaolinitic (mineral clay) soils with pH levels as high as 5.5 (Foy, 1974).

Acid soil conditions in developed countries can be managed by application of lime to raise the pH (Rao *et al.,* 1993). However in developing countries of the tropics this type of management is largely unavailable due to the high costs involved. Even when applied, liming is often not very successful because the sub-soil often remains unchanged (Rao *et al.,* 1993). Incorporation of lime to deep soils is technically difficult and expensive. Liming can also further decrease fertility, particularly phosphate and micronutrients such as zinc, and damage soil structure (Rao *et al.,* 1993). Plants grown on these limed soils often suffer from drought stress due to limited root growth in the acidic sub-soil (Foy, 1992).

1.2.2 Aluminium phytotoxicity

The easiest measurable effect of aluminium toxicity is inhibition of root growth, which can occur in less than one hour after treatment (Taylor, 1991). The root apices are thought to be the site of aluminium toxicity (Ryan *et al.*, 1993). Treatment of mature root does not cause inhibition of root growth, however treatment of the root apex causes inhibition of growth (Ryan *et al.*, 1993).

The high toxicity of aluminium is thought to be due to its high binding affinity with a wide variety of apoplastic and cellular components for example, proteins, DNA, RNA, ATP, lipid membranes and tubulin (Haug, 1984; Haug *et al.,* 1994; Matsumoto *et al.,* 1976; Clarkson, 1967; Wagatsuma, 1983; Sampson *et al.,* 1965). There are a

number of proposed mechanisms for aluminium toxicity. These include disruption of cell wall extensibility by binding to cell wall proteins or replacement of ions, disruption of cell membrane and nutrient transport by lipid peroxidation (Foy *et al.,* 1978; Foy, 1988), binding to lipids or cell membrane proteins (Wagatsuma, 1983), competition and blocking of cation transport sites (Huang *et al.,* 1996; Rengal and Elliot, 1992; Gassman and Schroeder, 1994) and disruption of cell metabolism by affecting ion transport or by directly binding to a variety of proteins, DNA, RNA or other important cell components (Haug, 1994; Rengal, 1992; Taylor, 1990; Delhaize and Ryan, 1995).

Evidence for many of these proposed mechanisms is so far unavailable or limited. It is still not known how much or how fast aluminium is able to enter the symplast, or the relative importance of the symplastic and apoplastic sites of attack for aluminium (Delhaize and Ryan, 1995). Most of the work attempting to measure aluminium concentration in root cells has hit problems trying to separate aluminium bound to the cell wall and membrane from the aluminium in the symplast (Reid *et al.,* 1995; Zhang and Taylor, 1990; Archambault *et al.,* 1996a).

1.2.3 Brachiaria species.

Brachiaria is a pan-tropical genus of grasses, with 60 out of approximately 100 species native to Africa (Parsons, 1972), and the rest mainly in the tropics of the eastern hemisphere. *Brachiaria decumbens* Stapf. (also known as Signal grass) was originally native to East Africa found in moist grasslands and savanna woodland. *Brachiaria ruziziensis* Germain & Evrard (also known as Ruzigrass or Congo Signal grass) is native to the Congo, Zaire, Rwanda and Burundi, where it occurs as a weedy colonist after forest clearance.

Brachiaria decumbens is limited in its distribution to latitudes between 27° north and south of the equator, and between sea level and 1750m above sea level (Skerman and Riveros, 1990). *Brachiaria decumbens* is adapted to a range of ecological

conditions. It thrives in both open grassland and under partial shade in open savanna woodland (Judd, 1979). *Brachiaria decumbens* optimum temperature is 30 to 35°C although it can survive frost. This low temperature sensitivity enables *Brachiaria decumbens* to enjoy a long growing season from spring to autumn (Skerman and Riveros, 1990). *Brachiaria decumbens* does not grow well in areas with dry spells lasting longer than 4 to 5 months, although during dry spells shorter than 4 months it grows much better than species such as *Digitaria decumbens*, *Panicum maximum* and *Brachiaria mutica* (CIAT, 1978). Despite *Brachiaria decumbens* drought tolerance, it prefers wet tropics with optimum rainfall in the region of 1500mm (Skerman and Riveros, 1990).

Brachiaria decumbens grows well in a wide range of soil types including infertile soils, acid soils, shallow soils and aluminium toxic soils, however maximum growth requires growth in fertile soils (Skerman and Riveros, 1990). In comparison *Brachiaria ruziziensis* requires well-drained fertile soils, although it will tolerate acidic soil conditions (Skerman and Riveros, 1990). *Brachiaria ruziziensis* is fairly drought tolerant, similar to *Brachiaria decumbens*, however it prefers areas with greater than 1000mm annual rainfall. *Brachiaria ruziziensis* is also far more temperature sensitive than *Brachiaria decumbens*, with optimum day/night temperatures of 33 and 28 °C respectively. *Brachiaria ruziziensis* growth is severely limited by temperatures as low as 24°C, and is killed by frost. Its growing season is therefore much shorter, lasting only the summer period (Skerman and Riveros, 1990). *Brachiaria ruziziensis* is usually only found between 1000 and 2000m above sea level.

Use of *Brachiaria* species for animal grazing and feed is widespread in the humid and sub-humid tropical regions, particularly South America where *Brachiaria decumbens* is one of the most widely sown fodder species (Thomas and Grof, 1986; CIAT, 1998). These grasses are especially important in areas with acid soil conditions and savannas, where they have increased the livestock production five to fifteen-fold. *Brachiaria* species are particularly adapted to acidic soil conditions and

its related stresses such as low nitrogen and phosphorus availability, low pH and mobilisation of toxic metals (Botrel *et al.,* 1990).

Despite the excellent growth of *Brachiaria* species in acidic and neutral infertile soils, their use is restricted to areas with a dry season less than 6 months and land below 2000m, where low temperatures do not limit their growth and competitive ability (Bogdan, 1977; Thomas and Grof, 1986). *Brachiaria decumbens* is better adapted to drought than *Brachiaria* (Rao *et al.,* 1993). Both *Brachiaria decumbens* and *Brachiaria ruziziensis* grow poorly on waterlogged or wet soil conditions (Hare *et al.,* 1999). *Brachiaria ruziziensis* productivity is particularly affected if exposed to regular periods of waterlogging (Hare *et al.,* 1999).

1.2.3.1 Low pH soil adaptation

Brachiaria species have a number of attributes which make them well adapted to both the low nutrient levels and toxic metals found in low pH soils. These include plasticity in carbon allocation between roots and shoots, which allows greater root growth at the expense of shoot growth in nutrient limited conditions, and efficient acquisition of the nutrients N, P, Ca and Mg through very large, extensively branched root systems and associated mycorrhizal systems (Rao *et al.*, 1992). They also exhibit high phytase and acid phosphatase activity and have low internal and external nutrient requirements for maximum growth (Li *et al.*, 1997).

In *Brachiaria decumbens,* nitrogen is absorbed in the nitrate rather than ammonium form (CIAT, 1984). The uptake of nitrate in preference of ammonium may be significant in *B. decumbens* adaptation to acid soils and aluminium toxicity. Uptake of ammonium results in the exudation of protons to balance the uptake of the positive charge on NH_4^+. This would further acidify the rhizosphere and soil solution around the roots.

The large extensively branched root systems and mycorrhizal associations of both *Brachiaria decumbens* and *Brachiaria ruziziensis* give very efficient uptake of

nitrogen (Rao *et al,* 1993). Species differences have been shown in the biological fixation of nitrogen by associate microbes as a percentage of total nitrogen uptake of *Brachiaria decumbens* and *Brachiaria ruziziensis*. Only 9% nitrogen uptake in *Brachiaria ruziziensis* is from symbiotic microbial fixation, compared to 40% in *Brachiaria decumbens* (Boddey and Dobereiner, 1988). This may be significant in explaining the better adaptation of *Brachiaria decumbens* to acid soils than *Brachiaria ruziziensis.*

One of the adaptations of *Brachiaria decumbens* to acid soil conditions is its enhanced ability to take up phosphate. *Brachiaria decumbens* has been shown to exude relatively high levels of phytase, which is a major component of the phosphate acquisition mechanism (Li *et al.*, 1997). Exposure of *Brachiaria decumbens* to low phosphate conditions causes an increase in phytase secretion and activity and an increase in general acid phosphatase activity (Li *et al.*, 1997). Out of the 18 species they tested, *Brachiaria decumbens* was found to have the highest phytase activity.

Both *Brachiaria decumbens* and *Brachiaria ruziziensis* have high calcium uptake efficiencies, due to the extensive branching of roots, leading to more root tips, which are the main site of calcium uptake (Hausler and Rao, unpublished, cited in Rao *et al,* 1993; Haussling *et al.,* 1988).

Correa and Reichartt (1995) found that growth of *Brachiaria ruziziensis* grown in field conditions was unaffected by treatment with aluminium, classing *Brachiaria ruziziensis* as an aluminium accumulator due to the accumulation of aluminium in the roots. They also noted that aluminium treatment did not cause a decrease in magnesium or calcium uptake. *Brachiaria decumbens* has been shown to be even more tolerant to aluminium and low pH than *Brachiaria ruziziensis* (CIAT, 1998).

1.2.3.2 Economical and agricultural benefits of *Brachiaria species*

Brachiaria decumbens has become one of the most economically important of the *Brachiaria* species due to its greater ability to grow well in poor acidic soil

conditions. *Brachiaria ruziziensis* is far less economically important or widely used due to slightly lower tolerance to acid soils conditions, where it is found to reduce in productivity over time (Kumar *et al.,* 2001).

In many parts of Brazil, such as the Nhecolandia sub-region, beef production is the main economic activity. The native grass species are subject to high seasonal variation in quality and availability and are of low nutritional value. Productivity in most Brazilian beef and dairy herds is low and depends almost totally on the pasture, which is dependent on soil conditions and pasture species composition.

Brachiaria decumbens was introduced into Brazil in 1976 from Australian seeds (Driermeier *et al.,* 1999). In soils with low pH and low available phosphorus the introduction of acid soil tolerant grass and legume varieties have increased beef production 10-15 fold with only modest phosphorus fertiliser inputs (Oberson *et al.,* 1999).

Field studies by Comastri and Pott (1998) looking at over eighty potential fodder species to evaluate the best species for economic benefit found that *Brachiaria decumbens* and *Brachiaria humidicola* were the best two grass species. Further studies by Dubeux *et al.* (1997) have found that productivity of *Brachiaria decumbens* pastures remains much higher than normal productivity levels in the region, and recommend use of *Brachiaria decumbens* for increased productivity).

1.2.3.3 Use as a animal feed

Despite the increased production of biomass yield by switching to growing *Brachiaria decumbens* on acidic soils, the nutritional quality is still low due to the low availability of various nutrients, such as N and P, in acidic soil conditions (Reis *et al.,* 1999). This is demonstrated by the negative effect on phosphorus balance in sheep fed with *Brachiaria decumbens* hay (Reis *et al.,* 1999). The nutritional value of *Brachiaria decumbens* (N and P content) can be increased by phosphorus fertilisation and inoculation with arbuscular mycorrhizal fungi (Carneiro *et al.,*

1999). Nutritional value can also be increased by growing mixed grass and legume pastures. However, in acidic soil conditions legume species are often out-competed over time, leading to grass-only pastures (Morais *et al.,* 1998a). Much work has been carried out looking at the best management practices such as fertiliser use, crop rotation and use of legume/grass mixtures to maintain high productivity over time (Hare *et al.*, 1999; Oberson *et al.,* 1999; Vilela *et al.,* 1999; Morais *et al.,* 1998b; Abdallah *et al.,* 1999; Sarda *et al.,* 1998).

Recently there has been some concern over the use of *Brachiaria decumbens* and various other crops as animal feeds due to the effect on entero-hepatic function of cattle (Abdullah and Rajion, 1997). A study examining the occurrence of foamy macrophages in the liver of Brazilian cattle found that prior to the introduction in 1976 of *Brachiaria decumbens* there was no occurrence of foamy macrophages. The study indicates that foamy macrophages were caused by prolonged ingestion of *Brachiaria* species (Driermeier *et al.,* 1999). The secondary compounds responsible in *Brachiaria decumbens* are saponins, epi-sarsasapogenin and epi-smilagenin (Abdullah and Rajion, 1997).

As well as cattle feed, *Brachiaria* species have been used as green manures to help increase soil fertility and structure. *Brachiaria ruziziensis* has been shown to be one of the better green manure crops for maintenance of soil structure and aggregation (Silva *et al.*, 1998).

1.2.3.4 Environmental concerns

Recently concern has been growing in South America over the effect alien grasses are having on native cerrado (S. American savanna) communities. The Cerrado is an extensive area of savanna covering a large area of Brazil. This area of savanna includes a huge variation in habitat which grade from open grassland known locally as 'compo limpo', to grassland dotted with shrubs and small trees called 'compo sujo', to more densely wooded grassland called 'cerrado' and finally to open woodland savanna called 'cerrado tipico' or simply 'cerrado'. Cerrado tipico makes up the largest proportion of the savanna habitat and hence gives its name to the

Brazillian savanna region (The Cerrado). A large study looking at graminoid species distribution in and around the Pe-de-Gigante reserve near Sao Paulo has shown that *B. decumbens* and other alien species are very common and exert a very strong competitive effect on the native grass species (Pivello *et al.,* 1999a). The campo cerrado (more open type of savanna) are at greater risk of alien invasion due to their more open aspect allowing sunlight into the lower layers. Further studies on these more open savannas have shown that two of the four dominant species are alien grasses, one of them *Brachiaria decumbens,* making up 13 % absolute frequency (Pivello *et al.,* 1999b). Transects running from the middle of the reserve (less disturbed) to the edge showed no change in distribution of the four major species, indicating a ubiquitous distribution of these alien species in native savanna (Pivello *et al.,* 1999b).

1.3 Aims of this study

Most of the current research into aluminium toxicity has been carried out on a few relatively aluminium sensitive species such as *Triticum aestivum*, *Zea mays*, *Arabidopsis* and *Oryza sativa* (Delhaize and Ryan, 1995). These were chosen for their agricultural importance, ease of growth and due to the large amount of information already known about their physiology. However, even relatively aluminium tolerant cultivars such as wheat - Atlas are severely affected by relatively low concentrations of aluminium (less than 100 μM) (Ryan *et al.,* 1993).

The use of *Brachiaria decumbens* and *Brachiaria ruziziensis* in this project gives the opportunity to look at the effect of aluminium toxicity and potential tolerance mechanisms on species that are very tolerant to aluminium. So far very little work has been carried out on the physiology of these species, particularly with regard to the effect of aluminium toxicity. Pilot studies at CIAT have shown that both species are tolerant to concentrations of aluminium in excess of 100 μM, causing less than 50% reduction in relative root growth compared to 100% inhibition in root growth of

tolerant wheat and maize cultivars (Ryan *et al.,* 1993; Bartlett and Riego, 1972). It is also known that root citric acid levels in both *B. decumbens* and *B. ruziziensis* are high and may be involved in aluminium tolerance, however the role of other organic acids and exudation of organic acids has yet to be investigated fully (CIAT, 1998).

This study aims to:

- Examine the relative aluminium tolerance of *Brachiaria decumbens* and *B. ruziziensis* compared to *Triticum aestivum cv atlas* using a number of physiological markers.
- Investigate the rate at which aluminium affects plant physiology and compare to the rate of induction of potential defence mechanisms in *B. decumbens* and *B. ruziziensis*.
- Investigate the hypothesis that aluminium causes a lower decrease in nutrient uptake in the more aluminium tolerant species.
- Investigate the adaptation of *B. decumbens* and *B. ruziziensis* to acid soil environments
- Investigate the hypothesis that *Brachiaria decumbens* and *B. ruziziensis* increase production, tissue accumulation and exudation of organic acids in response to aluminium.
- Determine which organic acids are primarily involved as a potential aluminium defence mechanism.
- Investigate the hypothesis that aluminium increases peroxidase and superoxide dismutase activity as a potential aluminium defence mechanism.
- Investigate the hypothesis that aluminium increases the reductive capacity of the oxidative stress system by measurement of root tip ascorbate concentrations.

Chapter Two: Methods

2.1 Growth of plant material

2.1.1 Germination and growth of plant material

Brachiaria seeds were obtained from Prof. I. M. Rao, working at Centro Internacional de Agricultura Tropical (CIAT), Apartado Aereo 6713, Cali, Columbia. To reduce the infection by fungi *Brachiaria decumbens* and *Brachiaria ruziziensis* seeds were first surface sterilised. This was by washing for 1 minute in 70% ethanol, washing thoroughly with distilled water, then washing for 15 minutes in a 2% NaOCl (sodium hypochloride, bleach) solution containing 0.1% Triton X-100 (detergent) and then finally washing thoroughly with distilled water. *Triticum aestivum cv atlas* seeds were not surface sterilised, due to no fungal growth occurring during germination on *Triticum aestivum* seeds. Seeds were germinated in rolls of moist absorbent paper, allowing vertical growth of roots and shoots. After 7 days the seedlings were transplanted to hydroponic culture.

2.1.2 Hydroponic culture

Plants were grown on in aerated hydroponic nutrient solution (see Table 2.1 for details of the nutrient solution) at 27/21°C, 12-hour light/dark periods. The plants were supported over 600 ml polypropylene beakers (Fisherbrand) using a 12 cm x 12 cm polypropylene sheet (Stewart Metal Alloys Ltd) with ten 1.5 cm diameter holes (Figures 2.1 and 2.2). The plants were held in position by 1.5 cm foam discs slit to the centre. Pots were painted black to exclude light from roots. A light source was provided by two banks of four 58W 1.5 m fluorescent strip lights (Wirefield Lighting) providing 350 to 400 $\mu mol/m^2/sec$ of photosynthetically active radiation. The nutrient solution was changed every 2 to 3 days.

Table 2.1: Hydroponic nutrient solution used in all experiments, unless otherwise stated. Aluminium treatment was by addition of 0 – 800 μM aluminium chloride to this standard hydroponic solution.

Nutrient	Concentration (μM)
NH_4NO_3	300
Na_2SO_4	50
K_2SO_4	100
$MgSO_4$	100
$Ca(NO_3)_2$	150
KH_2PO_4	30
$MnSO_4$	5
$FeCl_3$	5
$NaMoO_4$	0.1
$ZnSO_4$	0.1
$CuSO_4$	0.1
H_3BO_3	0.5

The nutrient solution was chosen due to its low nutrient levels compared to other nutrient solutions such as Long Ashton nutrient solution. Nutrient levels were similar to those used in the CIAT hydroponic studies (CIAT, 1998), which were based on available nutrient levels in the local tropical acid soils. The nutrient solution was adjested to pH 4.2 by addition of 1 M hydrochloric acid. Nutrient solution was replaced every 2-3 days. The nutrient solution pH was found to rise from 4.2 to between 4.4 and 4.5 after bathing roots for 2 days.

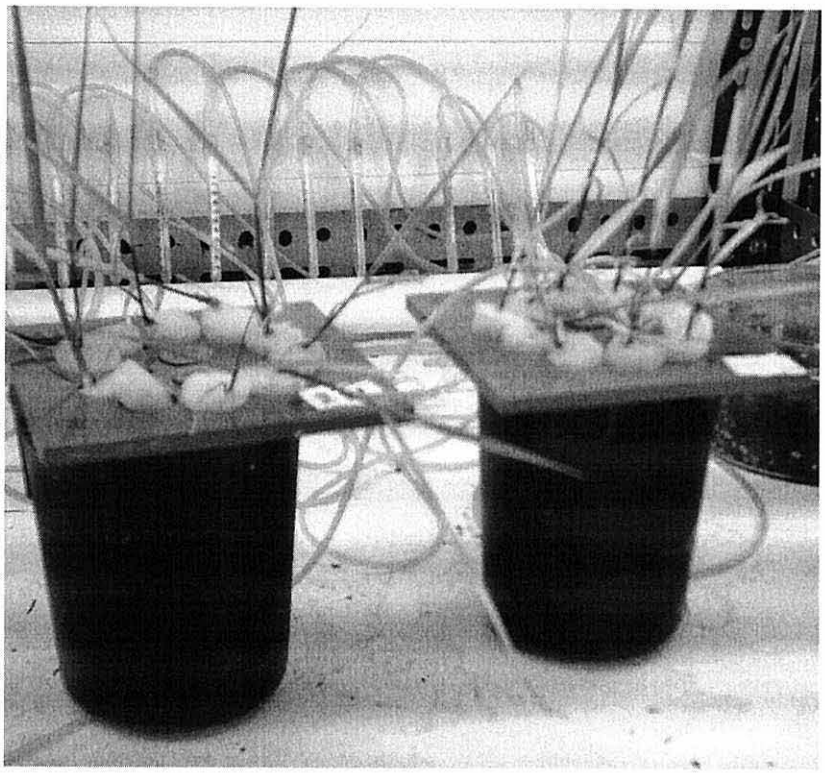

Figure 2.1: Hydroponic set up for growth and treatment of plants. *Brachiaria decumbens* on the left, *Brachiaria ruziziensis* on the right. The aeration system is visible in the background.

Figure 2.2: Growth chamber used for growth and treatment of plant material.

2.1.3 Use of Triticum aestivum cv atlas

After carrying out the initial experiments it was felt desirable to compare the performance of the two *Brachiaria* species with other species documented in the literature to give an indication of the relative aluminium tolerance of the two *Brachiaria* species in relation to other crop species and known aluminium tolerant and sensitive species/cultivars studied by other researchers. This comparison could not be achieved simply by looking at the scientific literature due to the different nutrient conditions and aluminium treatments used by other researchers. The amount of active aluminium in a solution is dependent on the nutrient concentrations, pH and the amount of aluminium used.

It was therefore necessary to introduce another species into the study. *Triticum aestivum* cv *atlas* was chosen due to the large amount of work already carried out on this species in relation to aluminium toxicity and general physiology. Not all experiments in the following chapters make use of *Triticum aestivum.* This is due to *Triticum aestivum* only being introduced part way through the Ph.D.

2.2 Organic acid analysis

2.2.1 Freeze rupture extraction

Roots were washed for 30 seconds in distilled water then transferred to clean distilled water for a further 30 seconds to remove loosely bound apoplastic cations and anions. Five 1cm pieces of root were cut for each sample, finely cut and placed in a labelled eppendorf vial. The eppendorf had been pierced at the bottom with a pin. The eppendorf was then dipped in liquid nitrogen to freeze the root tissue and thereby rupture the cell membranes. The pierced eppendorfs containing root tissue were placed over labelled non-pierced eppendorfs and each pair of eppendorfs centrifuged at 14000 rpm for 15 minutes (Hettich Zentrifugen EBA12, Gottingen). The cell contents were able to leak through the ruptured cell membranes and through

the aperture in the top eppendorf to collect in the bottom eppendorf. The cell walls and other non-soluble plant matter remained in the upper eppendorf (unless the diameter of the hole was too great). The aqueous sample, containing the organic acids, was then quickly frozen until analysis by capillary electrophoresis.

2.2.2 Analysis by capillary electrophoresis

In each replicate 1 µl of cell sap sample was diluted with 19 µl distilled water. 100 µM molybdate was added as an internal standard. Samples were kept frozen until assayed to prevent breakdown of organic acids in the sample by enzyme activity.

Samples were assayed by capillary zone electrophoresis using a Waters Quanta 4000 Capillary Electrophoresis system with a fused silica capillary (Supelco CElect FS75 CE column) with internal diameter 75 µm, external diameter 363 µm, total length 85 cm, and effective length (to detector) 77 cm. The CE capillary was conditioned daily by purging with distilled water for 10 minutes, followed by 1M NaOH for 30 minutes, then distilled water for 10 minutes, then with the carrier electrolyte for 30 minutes and finally running carrier electrolyte through at –15kV for 15 minutes. Between samples the column was purged with carrier electrolyte for 3 minutes.

For the separation and analysis the samples were run twice using two different carrier electrolyte systems. The first carrier electrolyte (Buffer 1) contained no calcium. This separated phosphate and malate, but oxalate was lost amongst the inorganic peaks. Buffer system 2, containing calcium did not allow separation of malate and phosphate but did shift the oxalate peak away from the inorganic peaks where it could be measured. Buffer 1 contained 2.5 mM pyromellitic acid, 15 mM Tris buffer and 1 mM **DOTAH (pH 11).** Buffer 2 contained 5 mM Tris, 2 mM trimellitic acid, 0.2 mM tetradecyltrimethylammonium bromide and 0.2 mM $CaCl_2$, pH was adjusted to 8.5 using 0.5 mM NaOH.

Samples were loaded using hydrostatic pressure injection (100 mm) for 30 seconds, and separation carried out at –15kV. After running samples using each buffer

system, 400 μM EDTA was added to each sample and the samples analysed again using the two buffer systems.

The integration of peak areas was carried out using ChromApex software. Peaks were identified by spiking samples with a range of known standards. Concentrations were calculated by comparing peak areas of organic acids with peak area of the 100 μM molybdate internal standard, with reference to calibration curves.

2.2.3 Aluminium effect on peak area and calibration curves

Experiments were carried out to look at whether the aluminium concentrations used during treatment of plant material affected peak areas of internal standards (tungstate and molybdate) and common organic acids. Solutions containing either 10, 20 or 50 μM of a range of organic acids (citrate, malate, oxalate, pyruvate, tartarate, succinate) and internal standards were prepared. These were analysed using capillary electrophoresis to give standard curves. The standards were mixed with varying concentrations of aluminium chloride and run to measure aluminium interference with analysis. Ethylenediaminetetraacetic acid (EDTA), which has a very high binding affinity with a number of metal cations, was added to these aluminium treated standards to investigate the effectiveness of EDTA in recovering organic acids bound to aluminium and other cations in samples.

It was found that treatment with aluminium reduced measurable peak areas significantly in a number of organic acids particularly citrate (80% reduction, Figures 2.3 to 2.11), but also to a lesser extent malate (10 to 30%) and oxalate (10 to 20%). Interestingly tungstate, originally used as internal standard, was also very badly effected by aluminium chelation (80% reduction in peak area), so was discarded as internal standard and replaced with molybdate, which was unaffected by aluminium. Also shown was the very high binding affinity of phosphate for aluminium, with 95% being bound up as aluminium phosphate (Figures 2.3 to 2.11).

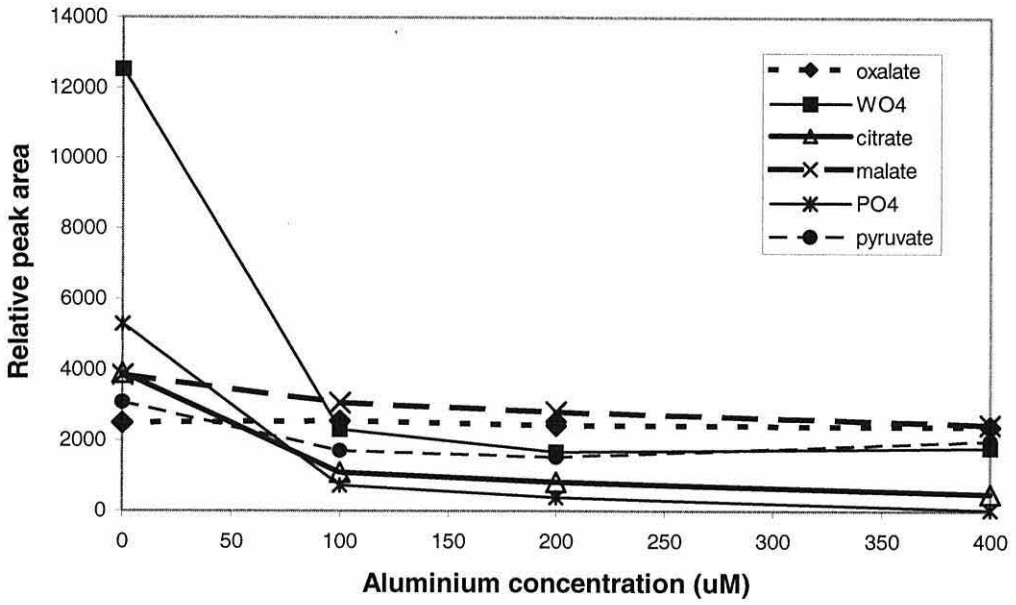

Figure 2.3: Effect of aluminium on peak areas of various 10 µM organic acid standards, citrate, malate, oxalate, pyruvate, phosphate (PO_4), and tungstate (WO_4). N=3.

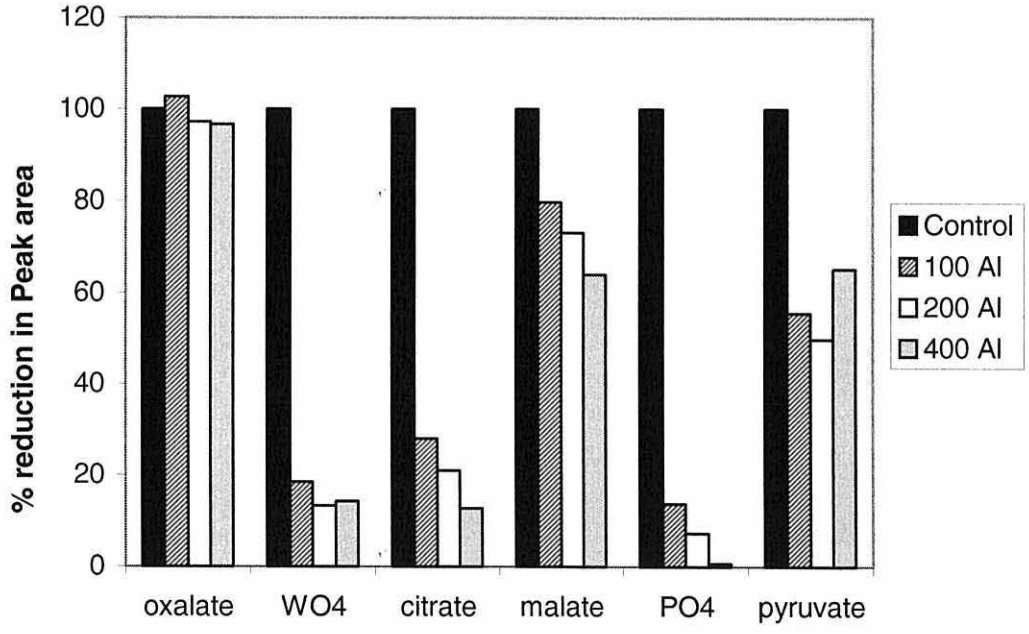

Figure 2.4: Percentage reduction in peak area of 10 µM standards caused by increasing aluminium concentrations in sample. N=3

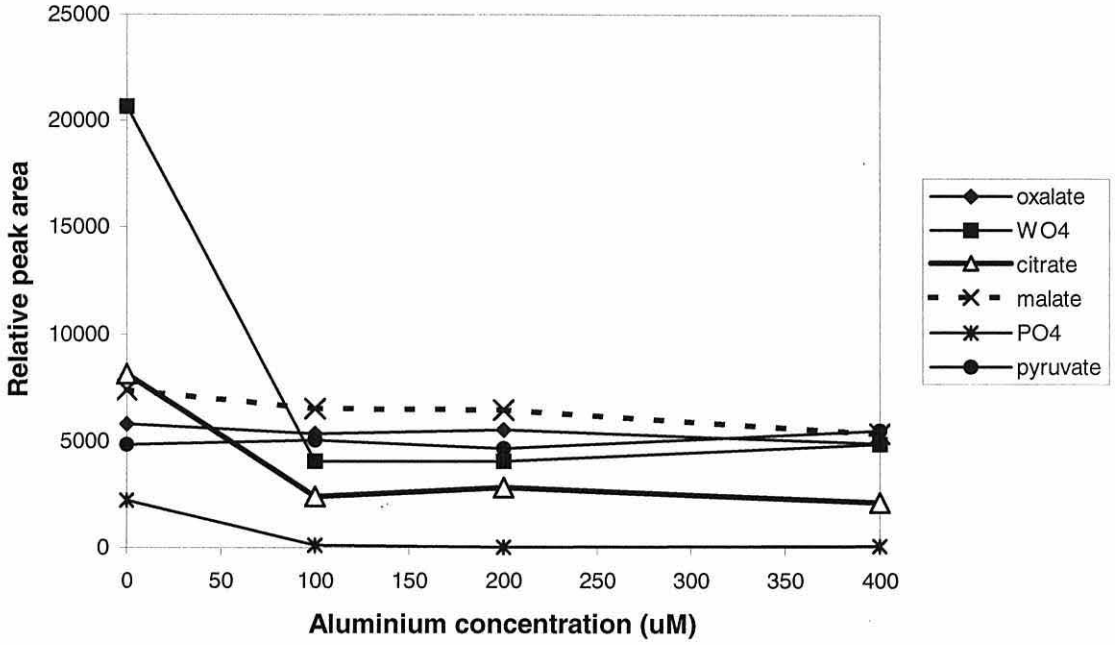

Figure 2.5: Effect of aluminium on peak areas of various 20 µM organic acid standards. N=3

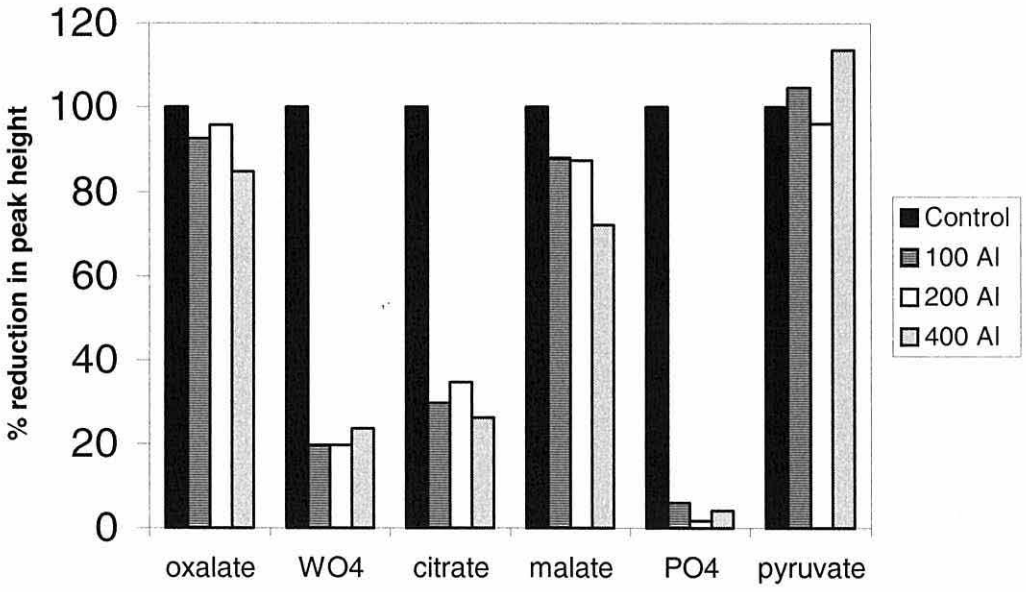

Figure 2.6: Percentage reduction in peak area of 20 µM standards caused by increasing aluminium concentrations in sample. N=3

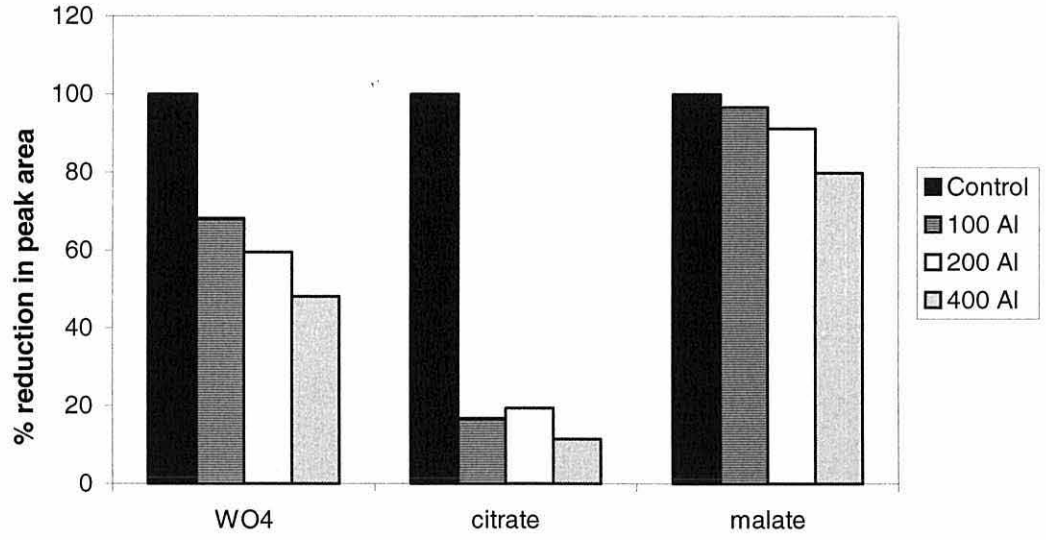

Figure 2.7: Percentage reduction in peak area of 50 μM standards caused by increasing aluminium concentrations in sample. N=3

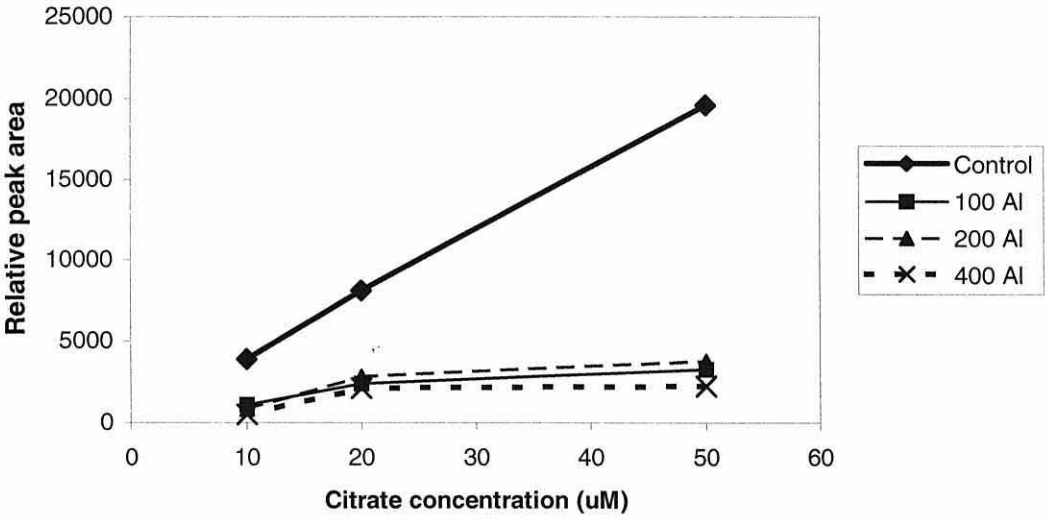

Figure 2.8: Effect of aluminium on the calibration curve of citrate

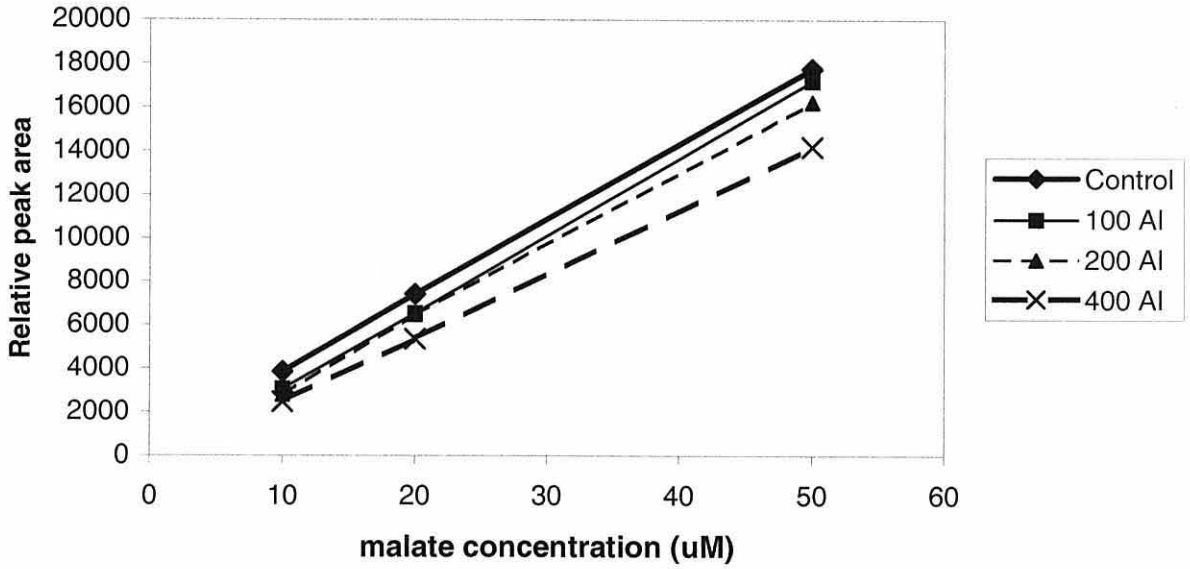

Figure 2.9: Effect of aluminium on the calibration curve of malate.

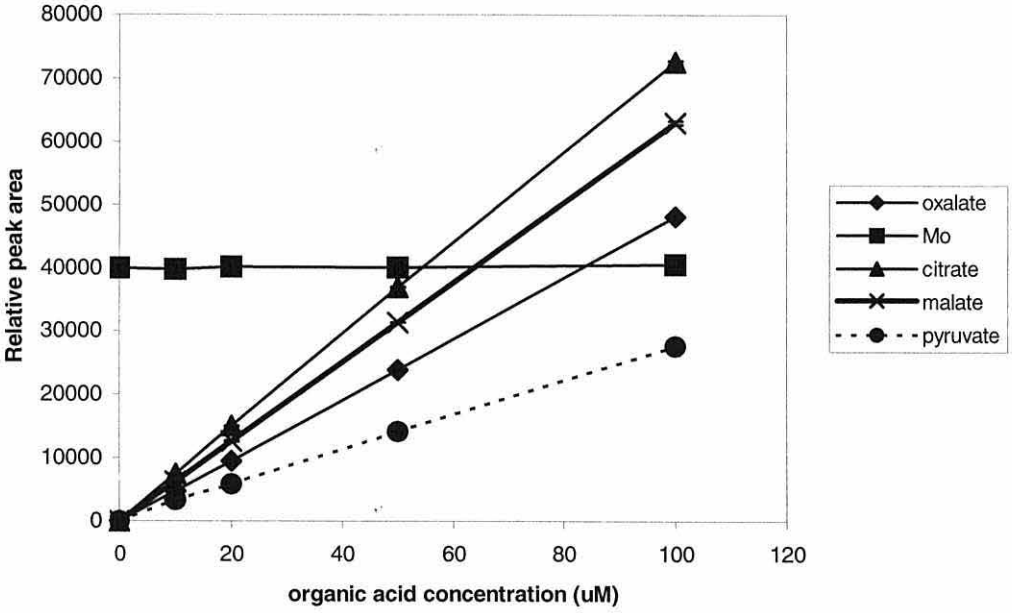

Figure 2.10: Ionic effect on molybdate internal standard.

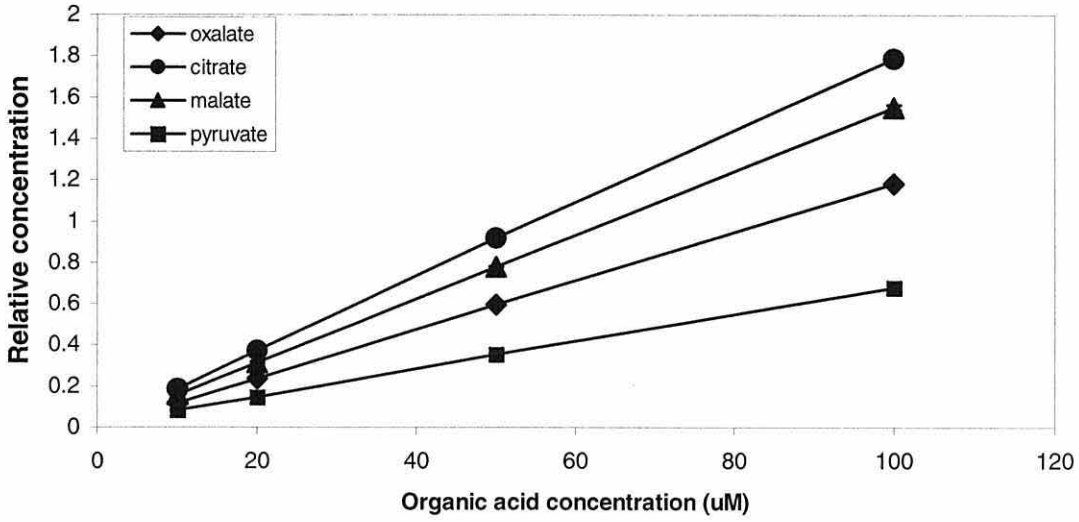

Figure 2.11: Calibration curve for citrate, malate, oxalate and pyruvate, using 100 μM molybdate as internal standard

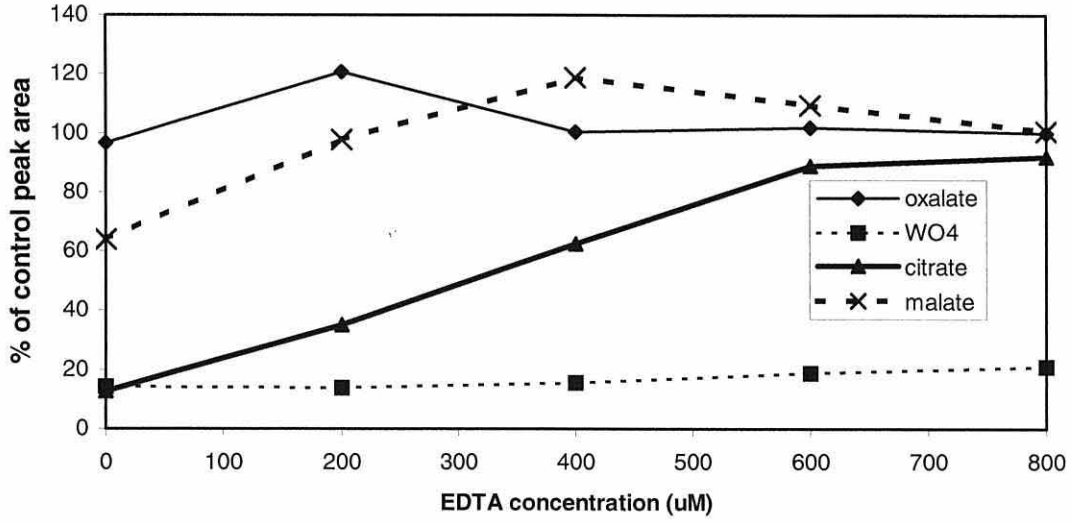

Figure 2.12: Effect of EDTA on recovery of organic acids, expressed as a percentage of control.

Addition of 600 μM EDTA was found to almost completely recover malate, citrate and oxalate peak areas (Figures 2.12 and 2.13). However in tissue samples concentrations greater than 400 μM EDTA had no further effect on increasing peak areas of key organic acids (Figure 5.12 and 2.13) and in fact obscured the malate peak (the peak for uncomplexed EDTA closely follows malate and phosphate peaks).

To check that the increase in organic acid peak areas by EDTA was due to the freeing of bound organic acid anions and not an ionic effect or other interaction, EDTA was added to organic acid standards in the absence of aluminium (Figure 2.14). EDTA had little or no effect on peak areas without presence of aluminium or iron, so addition of EDTA to control samples should have no deleterious effect on organic acid measurements (Figure 2.14).

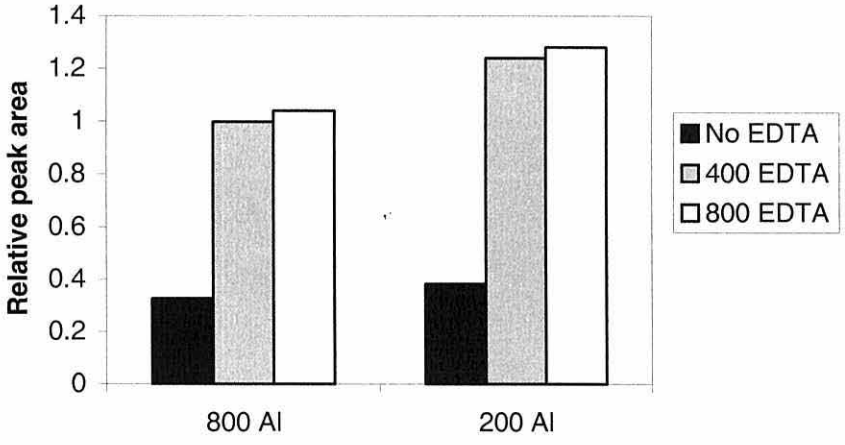

Figure 2.13: Effect of increasing EDTA concentrations on citrate recovery from aluminium treated samples.

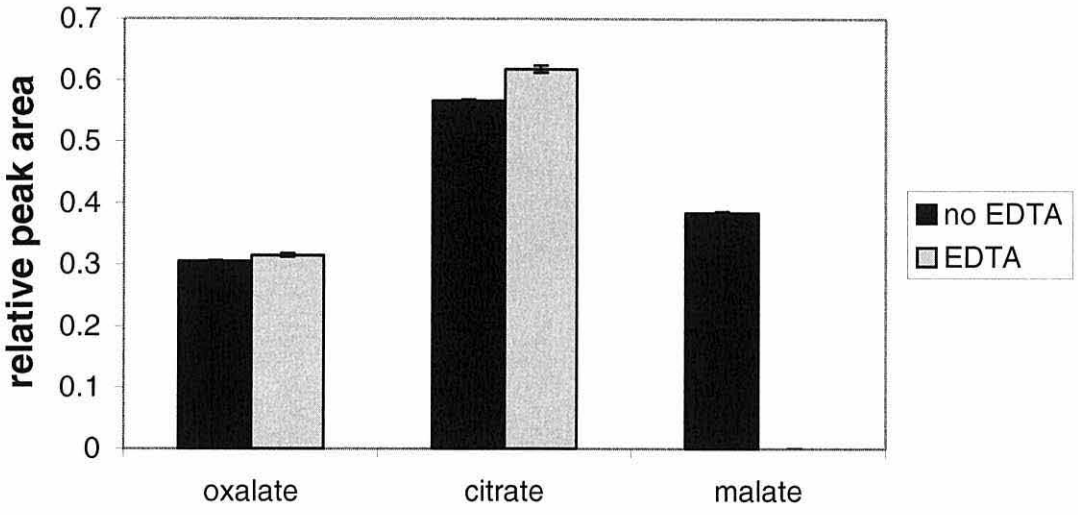

Figure 2.14: Effect of EDTA on the peak area of citrate, malate and oxalate.in the absence of aluminium. The malate peak was obscured by encroachment of the EDTA peak.

2.3 Statistical analysis

Data was statistically analysed using minitab version 13. All p-values result from one-way ANOVA, unless otherwise stated.

Chapter Three: Measurement of aluminium tolerance

3.1 Introduction

3.1.1 Inhibition of root growth

One of the earliest and easiest measurable affect of aluminium toxicity is the inhibition of root growth (Owenby and Popham, 1990; Llugany *et al.,* 1994; Sasaki *et al.,* 1994), which can occur within ten minutes of exposure to aluminium (Stass and Horst, 1995; Kochian and Jones, 1996). The root apices are thought to be the main site of aluminium toxicity (Ryan *et al.*, 1993), where aluminium affects dividing and expanding cells (Kochian, 1995; Vitorello and Haug, 1996). Research on *Nicotiana tabacum* tissue cultures found that only cells in the growing phase were affected by aluminium treatment (Vitorella and Haug, 1996). Selective treatment of the 1-2 mm root apices was found to inhibit root elongation, but treatment of root, excluding root apices, had no effect on root growth, indicating that the root apices are the sites of aluminium toxicity (Ryan *et al.*, 1993). Similarly aluminium treatment affects root hairs and apical and elongation zones - areas of high growth and cell division, whereas the mature root is unaffected (Ryan *et al.*, 1993). The apical and elongation zones accumulate higher levels of aluminium (Delhaize and Ryan, 1995).

The inhibition of root growth is thought at least initially to be due to the effect of aluminium on elongation rather than on the slower process of cell division (Jones *et al.,* 1995). Aluminium has been shown to affect microtubules, actin filaments and the cytoskeleton, causing structural damage and altering polymerisation patterns (Horst *et al.,* 1999a). The affect of aluminium on cytoskeletal development leads to alterations in cell elongation and morphology. One of the most noticeable marks of aluminium toxicity is the formation of club shaped root tips formed by loss of control of cell expansion (Kochian and Jones, 1996).

In some very tolerant species aluminium can stimulate root growth (Osaki *et al.*, 1997; Lian *et al.*, 1998). Species such as *Camellia sinensis*, *Melastoma malabathricum, Vaccinium macrocarpon, Polygonum sachalinense,* and *Melaleuca cajuputi* all significantly increase growth in response to levels of aluminium that would cause inhibition of root elongation in aluminium sensitive species such as *Triticum aestivum* (Osaki *et al.*, 1997; Lian *et al.*, 1998).

3.1.2 Callose

Another easily measured effect of aluminium toxicity is an increase in callose concentration. Callose (beta 1,3- Glucan) is a structural polysaccharide, very similar to cellulose (differing only in the glucose linkages), made up of a chain of glucose molecules with beta 1,3 bonding. It is produced in response to a variety of stresses including mechanical stress, tissue damage and wounding, infection, pollution, heavy metal exposure and aluminium exposure (Jaffe and Leopold, 1984; Zhang *et al.*, 1994). Induction of callose production can be rapid, occurring within 20 minutes of onset of the stress (Kohle *et al.*, 1985; Stass and Horst, 1995). Callose is produced by 1,3-ß-D-glucan synthase (callose synthase) at sites of membrane damage (Kohle *et al.,* 1985). Callose is thought to act as a defence and repair mechanism against damage to membranes, depositing rapidly around lesions caused by viral and fungal infections or aluminium toxicity, to help restrict infection or restrict movement of aluminium into the symplast (Bell, 1981; Shimomura and Dukstra, 1975; Marschner, 1991).

Kohle *et al.* (1985) have shown an important role for calcium in callose synthesis. Removal of apoplastic calcium inhibited callose synthesis. Kohle *et al.* (1985) suggest that callose synthesis in response to membrane lesions is mediated by increasing calcium influx from the apoplast through the lesion, with increasing cytosolic calcium causing an increase in callose synthesis.

Aluminium toxicity has been shown to induce callose synthesis in a number of species including *Camellia sinensis* (tea) (Lian *et al.,* 1998), *Triticum aestivum* (wheat) (Zhang *et al.,* 1994), *Glycine max* (soybean) (Stass and Horst, 1995; Massot *et al.,* 1999), *Zea mays* (maize) (Horst *et al.*, 1997), *Picea abies* (Norway spruce) (Wissmeier *et al.,* 1998), *Nicotiana tabacum* (tobacco) cell culture (Sivaguru *et al.*, 1999) and *Hordeum vulgare* (barley) (Budikova and Mistrik, 1999). In *Hordeum vulgare* cultivars, aluminium treatment was found to inhibit root growth and cause lesions in the apical zone. Callose formation was found around the lesion but not in the mature root tissues (Budikova and Mistrik, 1999).

Measurement of callose has been used as a sensitive tool for indicating aluminium toxicity (Wissmeier *et al.*, 1998), however it is also a good measure of relative aluminium sensitivity or resistance (Horst *et al.*, 1997; Massot *et al.,* 1999). Callose measurements correlated exactly with root growth inhibition data for thirty-two *Zea mays* cultivars treated with aluminium to test for aluminium sensitivity (Horst *et al.*, 1997). In the aluminium accumulating plant *Camellia sinensis* (tea) 800 μM aluminium was found to stimulate root elongation and caused no induction of callose synthesis (Lian *et al.*, 1998). Only at exceptionally high aluminium concentrations (8 mM) was callose synthesis induced (Lian *et al.*, 1998), indicating that toxic levels of aluminium are needed before callose synthesis is induced.

Callose decomposition can occur if the stress is removed. In one study, twenty-two hours after cessation of aluminium treatment callose levels in previously treated tissues were found to decrease (Wissmeier and Horst, 1995).

3.1.3 Chapter aims

This chapter investigates key physiological changes in response to aluminium treatment, including root growth, changes in callose concentration, biomass and photosynthetic activity. Measurement of these physiological responses will enable the identification of aluminium concentrations required to cause aluminium toxicity in the three species, and the relative aluminium tolerance of the two *Brachiaria*

species in comparison with *Triticum aestivum cv atlas.* The speed with which aluminium toxicity affects plant metabolism, growth and callose induction will be measured. This will allow comparison with the response of potential tolerance mechanisms such as organic acid induction and oxidative stress response in later chapters.

3.2 Methods

3.2.1 Aluminium dose response experiment

The lengths of the primary tap root of 21-day-old *Brachiaria decumbens, Brachiaria ruziziensis* and *Triticum aestivum cv atlas* plants were measured from the base of the plastic support to root tip and treatment with nutrient solution containing 0, 200, 400 or 800 μM aluminium chloride was initiated. Each treatment had five pot replicates, each pot containing five plants. Photosynthesis and stomatal conductance measurements were taken 24 hours after start of aluminium treatments for *Brachiaria decumbens* and *Brachiaria ruziziensis* using a portable infrared gas analyser (ADC). After 10 days the primary tap roots were re-measured, and *Brachiaria decumbens* and *Brachiaria ruziziensis* plants harvested for biomass measurements. For biomass measurements root and shoots were separated and oven dried at 65°C for 5 days in paper bags, then mass determined.

All three species were examined for callose induction. For each pot replicate five 1cm sections of root tips were excised and analysed for callose content (See below for method).

Root diameter, branching patterns and number of tips of *Brachiaria decumbens* and *Brachiaria ruziziensis* root systems were measured by scanning roots treated with 0 or 800 μM aluminium and analysing the images using 'WinRhizo' software.

3.2.1.1 Callose assay

Root tissue callose analysis was carried out as described in Kohle *et al.* (1985). The samples were stored in 200 µl ethanol (98% v/v) to kill the root tissue and reduce autofluorescence). The excess ethanol was removed and 200 µl of 1M NaOH added. The tissue was macerated using an eppendorf macerator to break down tissue and allow greater penetration of NaOH. The samples were then heated to 80°C for 15 minutes in a water bath to solublise the callose. After allowing to cool for 20 minutes, the samples were centrifuged at 14000 rpm for 5 minutes (EBA 12, Hettich Zentrifugen,W Krannich GmbH Co.). For each sample two 71 µl supernatant sub-samples were taken, one to measure callose and the other autofluorescence. To one sub-sample 142 µl 0.1% w/v filtered aniline blue was added, followed by 74 µl of 1M HCl and 210 µl of 1M glycine/NaOH buffer pH 9.5. To the other sub-sample distilled water was substituted for aniline blue to allow measurement of autofluorescence. Two blanks with aniline blue and two without were prepared by using 1M NaOH instead of supernatant. The sub-samples were then heated to 50°C for 20 minutes in a water bath to decolourise the aniline blue. The callose was then measured using a fluorimeter at 400 nm excitation and 510 nm emission, using distilled water to zero the fluorimeter. Relative callose concentrations were calculated by subtracting the autofluorescence measurements (no analine sub-samples) from callose fluorescence (with analine).

3.2.2 Root elongation time course experiment

Seven-day old *Brachiaria decumbens, Brachiaria ruziziensis* and *Triticum aestivum* seedlings were marked with rotring ink (an inert dye) approximately 0.75 cm from the root tips. The marks were kept small and applied with a fine brush to avoid damage to the roots. The distance from the mark to the root tip was measured using a binocular dissecting microscope (Stemi SV 11, Zeiss) with a graticule eyepiece. The roots were placed back in their pots and re-measured after 2 hours. Plants which did not grow were discarded. The *Brachiaria* species were then treated with 0, 200 and 400 µM aluminium (time point 0), and *Triticum aestivum* was treated with 0 and

200 μM aluminium (time point 0). Each treatment had 4 pot replicates, each pot containing 10 plants minus the number that were damaged by the marking process (minimum 6 plants per pot). The roots were then re-measured 2, 4, 6, 8 and 24 hours after beginning treatment with aluminium.

3.2.3 Callose time course experiment

Fourteen-day-old *Brachiaria decumbens* and *Brachiaria ruziziensis* plants were treated with 0 or 200 μM aluminium in standard nutrient solution. Three replicate root tip samples per time point per treatment were taken 0, 2, 4, 10, 24 and 48 hours after the start of the treatment. Each sample consisted of five 1cm root tip sections excised and stored in 200μl ethanol (98% v/v). Samples were then analysed for callose as described in Chapter Two.

3.3 Results

3.3.1 Aluminium dose response experiment

3.3.1.1 Root growth

Brachiaria decumbens, Brachiaria ruziziensis and *Triticum aestivum* showed very strong differences in root growth inhibition in response to increasing aluminium treatment (Figure 3.1). *Triticum aestivum* root elongation was completely inhibited by treatment with 200 μM aluminium, whereas *Brachiaria decumbens* was unaffected and *Brachiaria ruziziensis* was only 50% inhibited. Treatment with 400 μM aluminium caused approximately 40% inhibition of root elongation in *Brachiaria decumbens* and 75% inhibition in *Brachiaria ruziziensis*. 800 μM aluminium treatment caused almost total inhibition in root growth of *Brachiaria ruziziensis*, but only 60% inhibition in root growth of *Brachiaria decumbens*

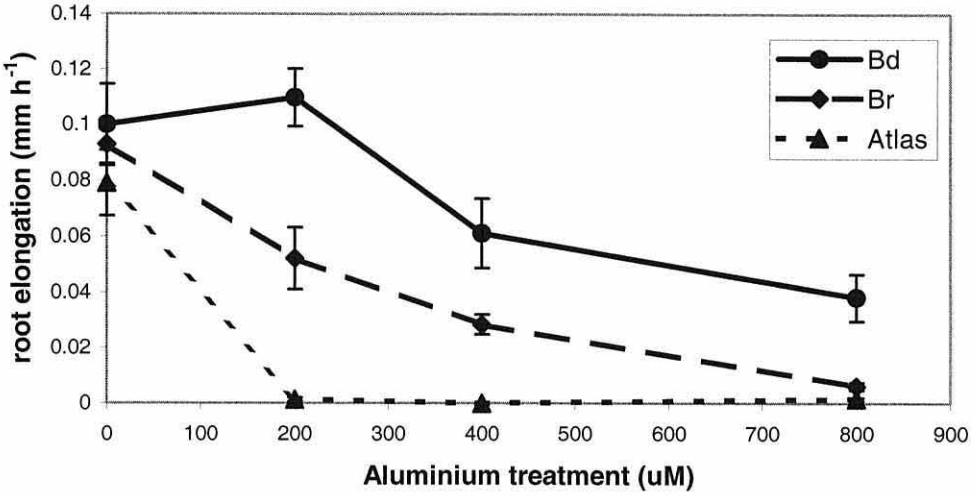

Figure 3.1: Root growth (mm h^{-1}) of *Brachiaria decumbens, Brachiaria ruziziensis* and *Triticum aestivum var atlas* after treatment with 0, 200, 400 or 800 μM aluminium. ± S.E. N=5

3.3.1.2 Callose content

There was a strong differential species response in callose concentration with increasing aluminium treatments. Treatment with 200 μM aluminium caused a 20 times increase in callose content in root tips of *Triticum aestivum* cv. Atlas ($p<0.001$) (Figure 3.2). Treatment with higher amounts of aluminium did not increase this further. *Triticum aestivum* callose concentration was significantly higher than callose levels in both *Brachiaria* species at all three aluminium treatments ($p=0.013$). Increasing aluminium treatment caused a significant increase in callose root tip concentration in both *Brachiaria* species ($P=0.022$ for Br and 0.027 for Bd). *Brachiaria ruziziensis* root tip callose content increased steadily with increasing aluminium, imitating almost exactly the effect of aluminium on root growth inhibition. However in *Brachiaria decumbens,* callose concentration increased after treatment with 200 μM aluminium whereas corresponding root growth was unaffected. It then steady increased with increasing aluminium treatment but not as greatly as in *Brachiaria ruziziensis*.

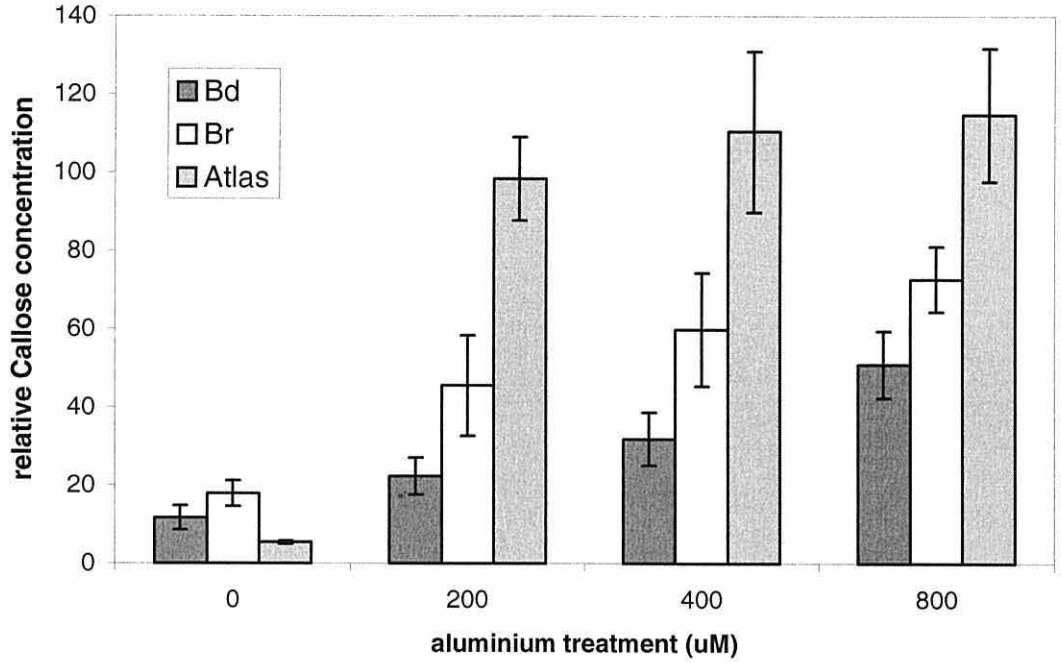

Figure 3.2: Relative callose concentration in root tips of *Brachiaria decumbens, Brachiaria ruziziensis,* and *Triticum aestivum var atlas* after a 10 day treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n=5.

3.3.1.3 Biomass

Aluminium caused a decrease in both root (Figure 3.3) and shoot (Figure 3.4) biomass in *Brachiaria decumbens.* However, due to the high variability in the data, the decrease in root biomass only becomes statistically significant ($p<0.05$) after treatment with 800 μM aluminium (Figure 3.3). *Brachiaria ruziziensis* root biomass is also gradually decreased by increasing aluminium treatment (Figure 3.3), however shoot biomass is unaffected (Figure 3.4)

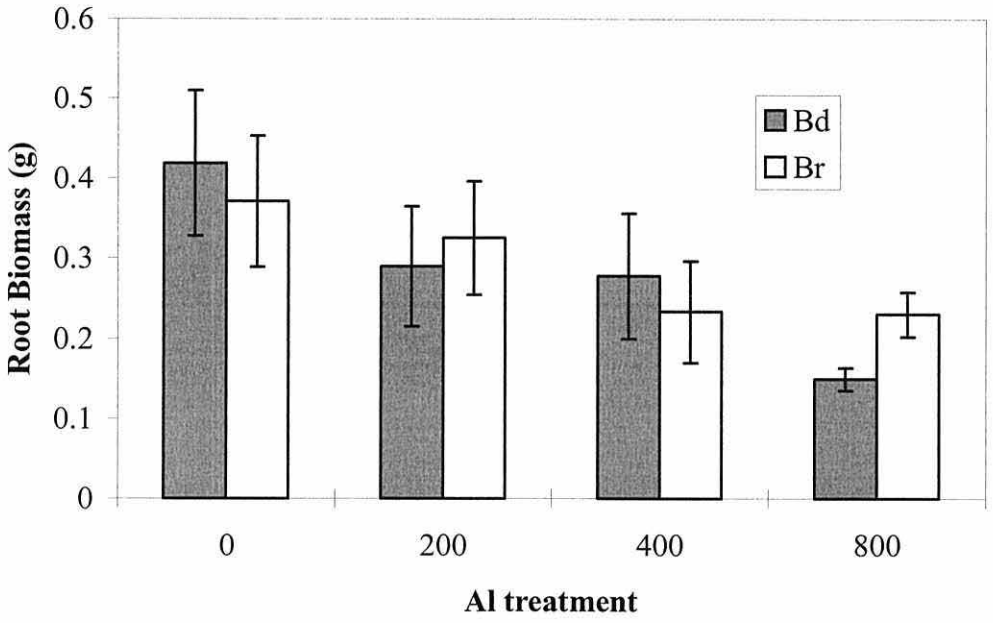

Figure 3.3: Root biomass (g/per 5 plants) for *B decumbens* and *Brachiaria ruziziensis* after 10 day treatment with 0, 200, 400 or 800µM aluminium. ± S.E. n=5.

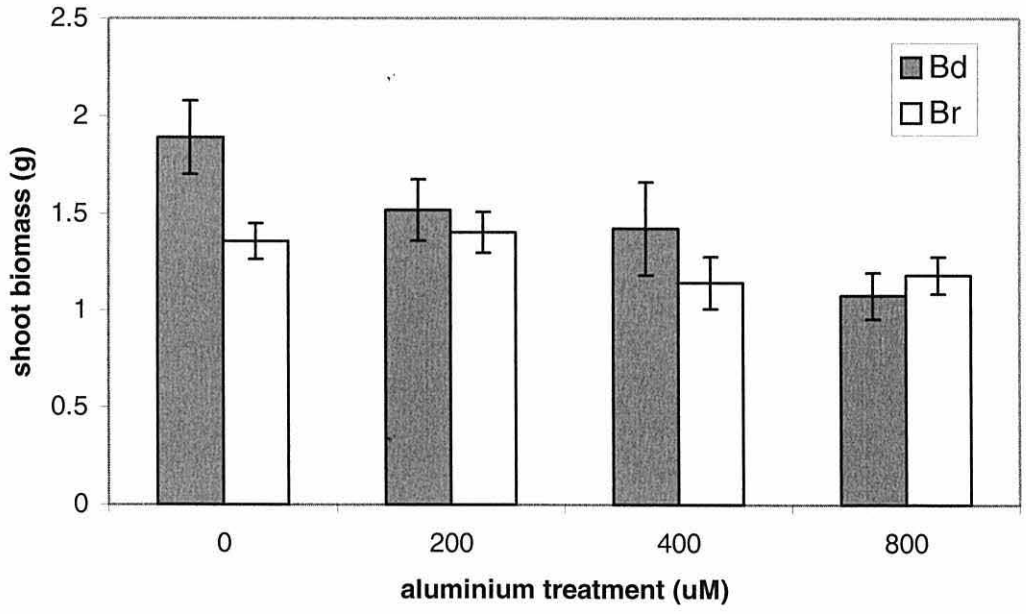

Figure 3.4: Shoot biomass (g/per 5 plants) for *B decumbens* and *Brachiaria ruziziensis* after 10 day treatment with 0, 200, 400 or 800µM aluminium. ± S.E. n=5.

3.3.1.4 Root Morphology

The 'WinRhizo' data shows *Brachiaria decumbens* has significantly greater root diameter ($p=0.009$) and less branching of roots and more root tips than *Brachiaria ruziziensis* (Table 3.1). Treatment with 800 μM aluminium caused a significant decrease in branching ($p=0.183$ Bd and 0.009 Br) and the number of root tips ($p<0.001$ for Bd and Br) in *Brachiaria decumbens* and *Brachiaria ruziziensis*. Average root diameter is significantly decreased by treatment with 800 μM aluminium in *Brachiaria decumbens* ($p=0.022$) but not in *Brachiaria ruziziensis*.

Table 3.1: *B. decumbens* (Bd) and *B. ruziziensis* (Br) root morphology. Average root diameter (mm), average number of forks per cm and average number of root tips per cm, with standard errors. n=7 and 10 for Bd and Br respectively. Plants treated with 0 or 800 μM aluminium.

	Average diameter, mm		*Forks per cm root length*		**Root tips per cm root length**	
	Mean	**S.E.**	**Mean**	**S.E.**	**Mean**	**S.E.**
Bd control	0.084	0.008	5.16	0.41	6.86	0.66
Bd 800 Al	0.056	0.005	4.37	0.15	1.55	0.04
Br control	0.056	0.003	5.64	0.32	5.77	0.29
Br 800 Al	0.054	0.001	4.00	0.11	1.51	0.03

3.3.1.5 Photosynthesis and stomatal conductance

Photosynthesis rates were similar in both *Brachiaria decumbens* and *Brachiaria ruziziensis* (Figure 3.5) and in both species was unaffected by 24 hours of aluminium treatment (p=0.055 in Bd, 0.721 in Br). Stomatal conductance was also unaffected by treatment with aluminium (p=0.535 in Bd and 0.51 in Br) (Figure 3.6). Stomatal conductance was almost twice as high in *Brachiaria decumbens* than *Brachiaria ruziziensis* (p<0.001).

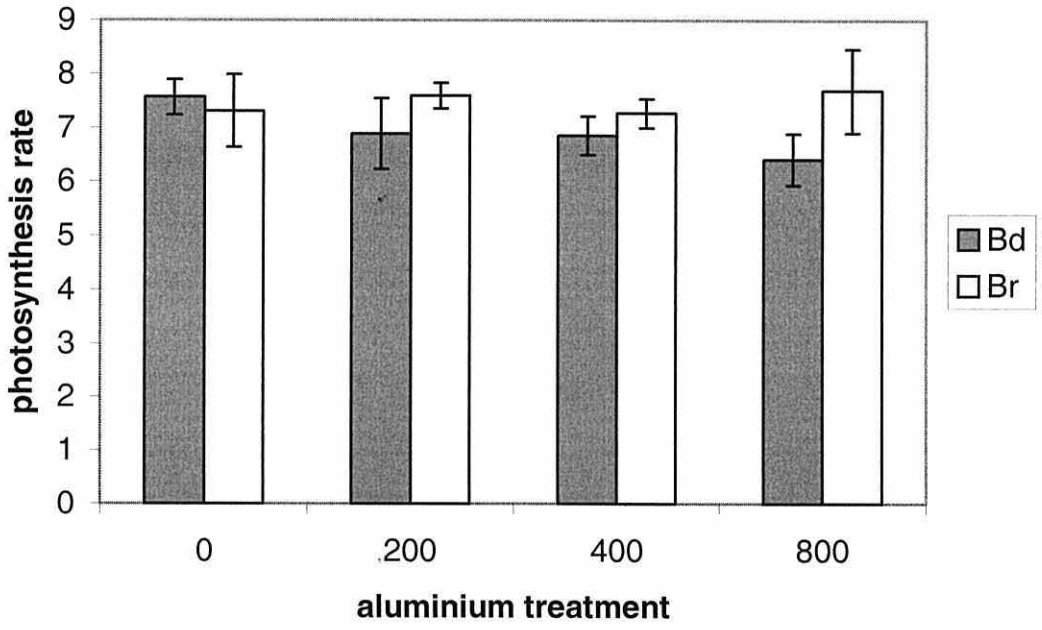

Figure 3.5: Photosynthetic rate in *Brachiaria decumbens* and *Brachiaria ruziziensis* after 24 hour treatment with 0, 200, 400 or 800 μM aluminium. ± S.E. n=4

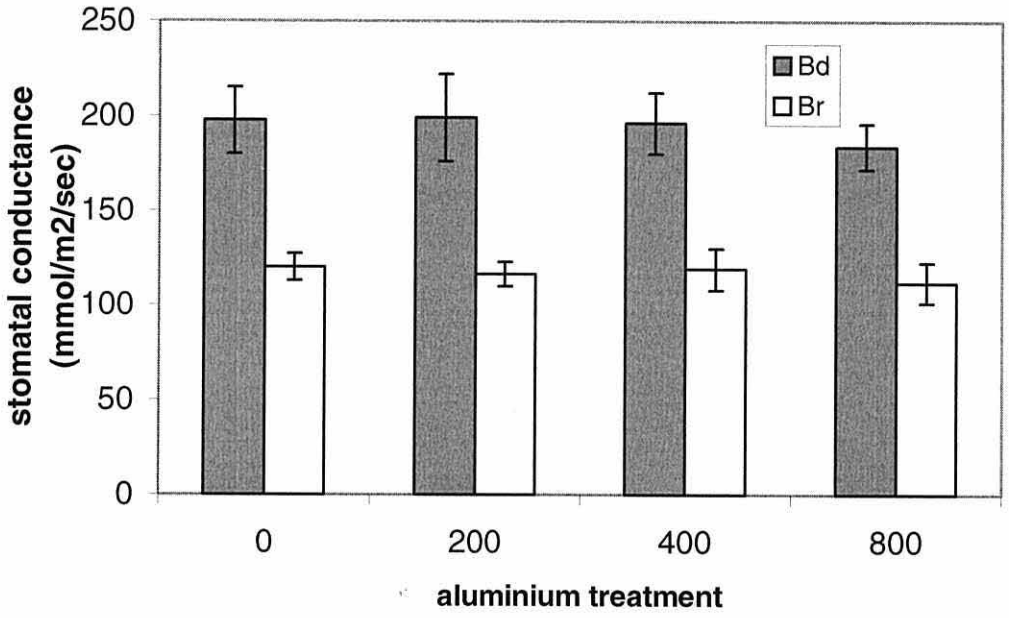

Figure 3.6: Stomatal conductance in *Brachiaria decumbens* and *Brachiaria ruziziensis* after 24 hour treatment with 0, 200, 400 or 800 μM aluminium. ±S.E. n=4

3.3.2 Root elongation time course experiment

The root elongation time courses show inhibition in root elongation between 4 and 6 hours of treatment with aluminium in *Brachiaria decumbens* (Figure 3.7) and after 6 hours in *Triticum aestivum* cv. Atlas (Figure 3.8). The data also showed a possible short-term fertilisation affect with 200 μM aluminium in *Brachiaria decumbens*. This agrees well with the initial root growth inhibition data (Figure 3.1). The data for *Brachiaria ruziziensis* is less convincing than for *Brachiaria decumbens* (Figure 3.9). There was a lower survival rate of the *Brachiaria ruziziensis* plants due to the marking process and mechanical damage caused by being repeatedly removed and replaced in their holders. The data indicates that the effect of increasing aluminium treatment on root growth occurs between 3 and 8 hours. A recovery of root growth in all three species can be seen between 8 and 24 hours. This is probably partly due to decreased handling and mechanical stress inflicted on the plants during this period. *Brachiaria ruziziensis* appears to be more prone to suffer mechanical damage than either *Brachiaria decumbens* or *Atlas*, more plants of which survived the experiment and grew better than *Brachiaria ruziziensis*.

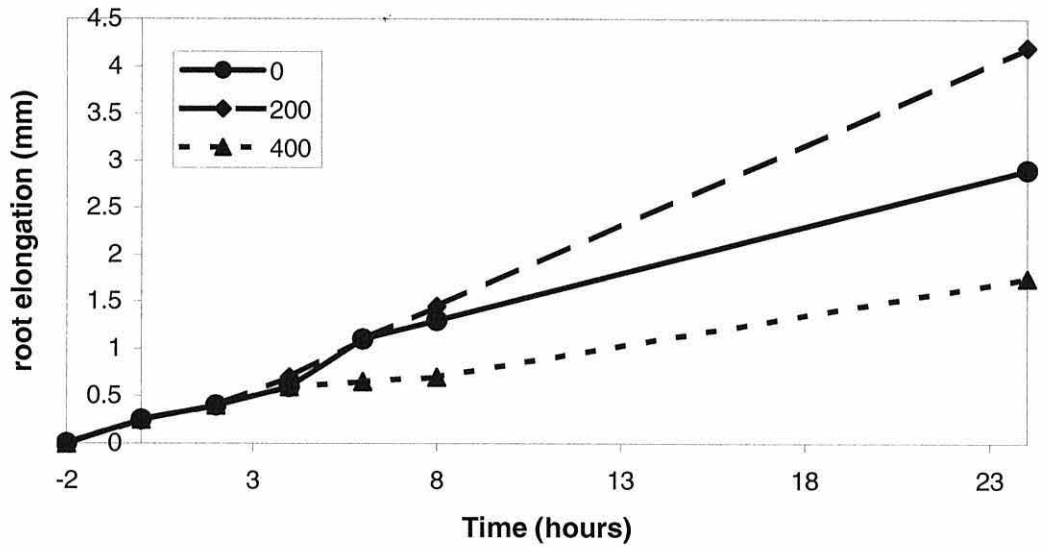

Figure 3.7. *Brachiaria decumbens* root growth over a 24 hour period, treated with 0, 200 or 400 μM aluminium at time point zero. n = 4.

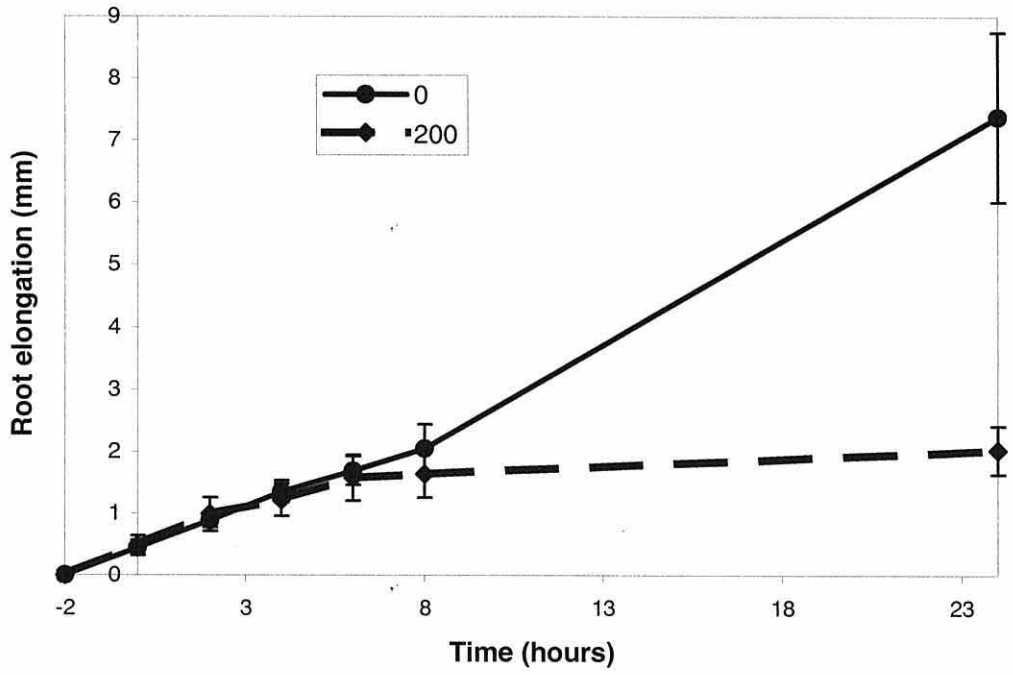

Figure 3.8. *Triticum aestivum* root growth over a 24 hour period, treatment with 0 or 200 μM aluminium commenced at time point zero. ± S.E. n = 4.

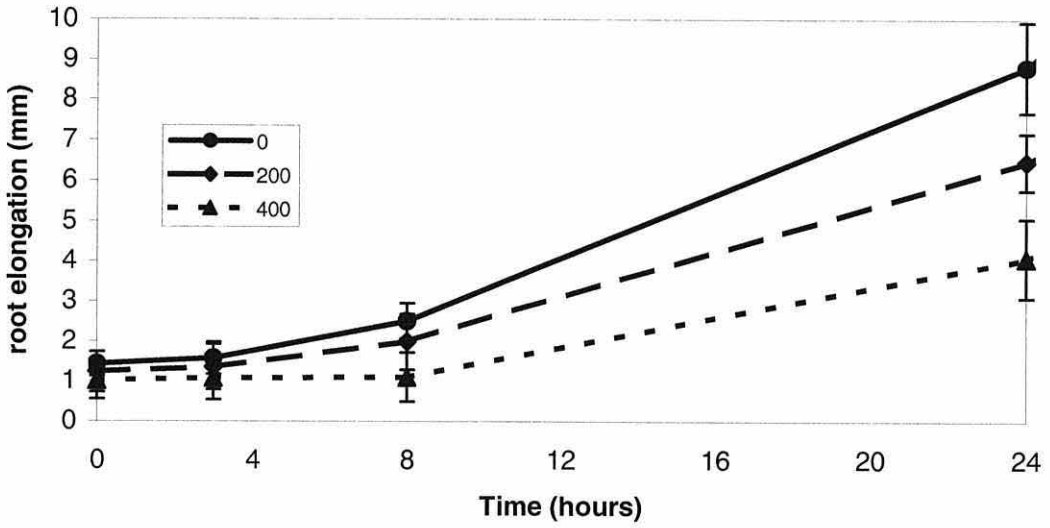

Figure 3.9: *Brachiaria ruziziensis* root growth over a 24 hour period, treatment with 0, 200 or 400 μM aluminium commenced at time point zero. ± S.E. n = 4.

3.3.3 Callose time course experiment

The *Brachiaria decumbens* callose induction time course experiment showed a huge induction of callose (2 – 3 times initial concentrations) between 2 and 4 hours of treatment with 200 μM and 400 μM aluminium (Figure 3.10). Callose synthesis is therefore occurring prior to inhibition of root growth in *B.* decumbens, which occurred between 4 to 6 hours after aluminium treatment. Callose root tissue concentration is increased equally by 200 μM and 400 μM aluminium treatment, whereas at 200 μM there was an aluminium treatment fertilization effect on root growth and root elongation is only inhibited at 400 μM aluminium.

Induction of callose in *Brachiaria ruziziensis* was very similar to that of *Brachiaria decumbens,* occurring between 2 and 4 hours after treatment (Figure 3.11). Callose induction in *Brachiaria ruziziensis* again pre-empts root growth inhibition.

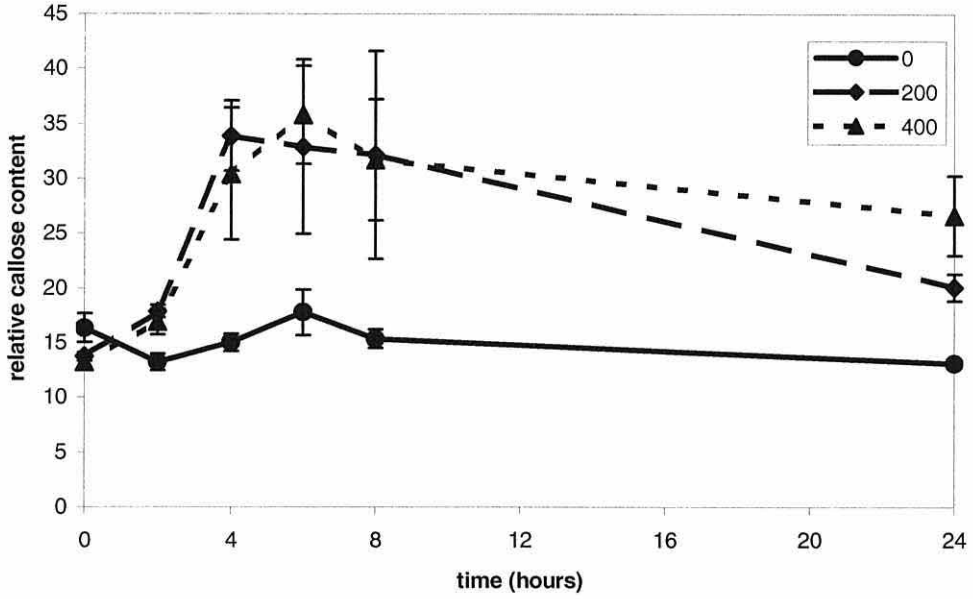

Figure 3.10: Induction of callose in *Brachiaria decumbens* root tips after treatment with 0, 200 or 400 μM aluminium. ± S.E. n=3

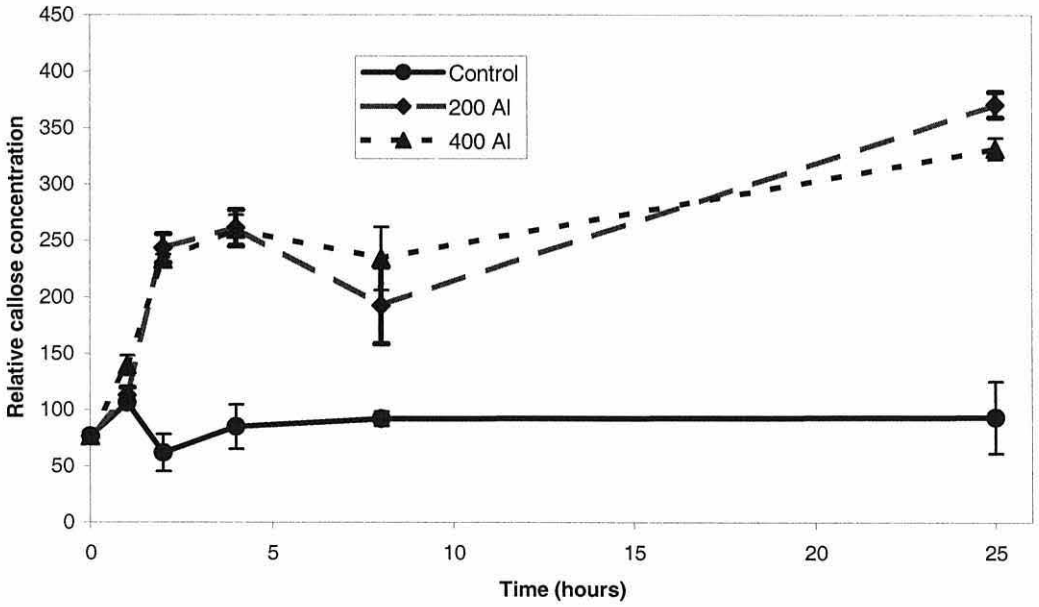

Figure 3.11: Induction of callose in *Brachiaria ruziziensis* root tips after treatment with 0, 200 or 400 μM aluminium. ± S.E. n=3.

3.4 Discussion

3.4.1 Aluminium dose response experiment

3.4.1.1 Root Growth

The root growth data shows *Brachiaria decumbens* to be completely unaffected by 200 μM aluminium, with root growth only decreasing after treatment with 400 and 800 μM. 800 μM aluminium is extremely high, field conditions would be much nearer 100 μM or less. *B. decumbens* is far more aluminium tolerant than *B. ruziziensis,* which was significantly affected by 200 μM aluminium. The much lower aluminium tolerance of *B. ruziziensis* than *Brachiaria decumbens* may partly explain why *B. ruziziensis* does not survive as well as *Brachiaria decumbens* in aluminium toxic field conditions, where aluminium exposure may be lower than the 200 μM level used in this experiment but aluminium exposure is continual and long-term.

The root growth data also shows just how much more aluminium tolerant both *Brachiaria* species are than conventional agricultural crops such as *Triticum aestivum.* Tolerant cultivars such as atlas are significantly affected by 20 to 50 μM aluminium treatment (Ryan *et al.,* 1993), making *Triticum aestivum* and other agriculturally important species an order of magnitude more aluminium sensitive than *B. decumbens.* At the other end of the scale *B. decumbens* is much more aluminium sensitive than *Camellia sinensis*, which is unaffected by 800 μM aluminium treatment (Osaki *et al.*, 1997; Lian *et al.*, 1998).

3.4.1.2 Callose

Callose has been shown to be induced by a number of environmental factors including aluminium toxicity (Jaffe and Leopold, 1984; Zhang *et al.*, 1994). Callose is laid down around lesions of membranes damaged by aluminium as a defensive mechanism (Budikova and Mistrik, 1999). The induction of callose in the root tips of *Brachiaria decumbens, Brachiaria ruziziensis* and *Triticum aestivum* indicates the

occurrence of tissue damage to the root tips caused by aluminium treatment. The much higher callose induction in *Triticum aestivum* indicates much higher levels of injury caused by aluminium than in either *Brachiaria* species.

The callose data agrees strongly with the root elongation data in determining relative aluminium sensitivity of the three species tested. However, there is a clear disagreement between callose induction and root growth inhibition data in determining at what dose aluminium becomes toxic to *B. decumbens,* with induction of callose synthesis appearing to be a more sensitive indicator of aluminium toxicity than root growth.

3.4.1.3 Biomass

The decrease in root and shoot biomass in *B. decumbens* follows the pattern of callose response rather than root growth inhibition, which was unaffected by 200 μM aluminium. Decreased root biomass while maintaining root growth at concentrations of 200 μM aluminium may be partly due to a decrease in average root diameter with aluminium treatment. The root morphology data shows this to be the case. Treatment with 800 μM aluminium decreases root diameter in *Brachiaria decumbens*. There is also a decrease in root branching with aluminium treatment, which would also decrease root biomass. However, it must be remembered that growing plants in hydroponic culture affects root morphology and these root morphological changes in response to aluminium treatment may not be replicated in a soil system.

Brachiaria ruziziensis also showed a decrease in root biomass in response to increasing aluminium treatment, however shoot biomass was unaffected by aluminium treatment. The difference in shoot biomass response to aluminium between the two species is probably due to the higher investment in shoots by *Brachiaria decumbens* in control conditions. *Brachiaria decumbens* had a significantly lower root:shoot ratio than *Brachiaria ruzuziensis* in the control solution. This may indicate a more efficient nutrient uptake system.

3.4.1.4 Photosynthesis and Stomatal Conductance

Aluminium has been shown to affect photosynthesis and stomatal conductance in a number of plant species (Pereira *et al.,* 2000; Ridolfi and Garrec, 2000; Moustakas *et al.,* 1997). In a variety of citrus species, aluminium treatment has been shown to significantly decrease photosynthesis, but had no effect on stomatal conductance (Pereira *et al.,* 2000).

In Fagus sylvatica aluminium was found to decrease calcium and magnesium and increase potassium shoot concentrations. Aluminium treatment caused a decrease in stomatal function. This effect was amplified by calcium and magnesium deficiency and appeared to be linked to reduced potassium flux in and out of guard cells (Ridolfi and Garrec, 2000).

Aluminium treatment has been found to disrupt mesophyll structure and spatial arrangement of chloroplasts (Moustakas *et al.,* 1997). Aluminium treatment was also found to disrupt chloroplast ultrastructure, causing degradation of photosystems and other photosynthetic structures. These effects were deduced to be indirect due to no detectable aluminium in the leaves. The change in mesophyll structure was attributed to swelling of leaf tissue due to changes in membrane permeability, and the break down to photosynthetic apparatus due to changes in nutrient uptake patterns (Moustakas *et al.,* 1997).

Stomatal conductance and photosynthetic rate were unaffected in both *Brachiaria ruzuziensis* and *Brachiaria decumbens* with increasing aluminium treatment. This is not surprising as roots rather than shoots are the area of attack for aluminium toxicity. Shoot growth and metabolic processes are unlikely to be directly affected by aluminium, which has a very low rate of transport through the plant and accumulation in above ground biomass (Chapter Four looks further at aluminium accumulation in shoot and root biomass). Photosynthetic rate will probably only decrease as general plant metabolism becomes inhibited due to decreased nutrient

uptake and decreased metabolism of root tissue starts to cause deficiencies in key metabolites. Photosynthetic capacity will also be decreased in relation to control plants due to decreases in shoot growth and leaf size caused by aluminium toxicity.

The much lower stomatal conductance in *Brachiaria ruziziensis* than *Brachiaria decumbens* while maintaining similar photosynthetic activity indicates that *Brachiaria ruziziensis* has better water use efficiency and so in theory would be better adapted to drought conditions.

3.4.2 Time course experiments

The time course data showed callose induction occurs prior to root growth inhibition in both *Brachiaria* species, with callose induction occurring after 2 hours and inhibition of root growth after 4 hours. This implies that root damage is occurring prior to inhibition of root growth. These two processes are very likely unrelated in terms of mechanism of induction, although callose accumulation may play a part in decreasing root growth by causing a rigidification of cell wall structure, decreasing structural elasticity, which may in turn hinder root growth and elongation.

The root growth time course also shows a clear stimulation effect of 200 μM aluminium on *Brachiaria decumbens.* This corroborates the root growth dose response data, which found a similar effect. This level of aluminium treatment is relatively high in comparison to typical field levels, which are more in the region of 100 μM aluminium. This gives some indication of why *Brachiaria decumbens* thrives in high aluminium soil conditions.

The stimulation of growth by aluminium could be due to a number of factors. For example increased uptake of key nutrients such as nitrogen, phosphate and potassium by *Brachiaria decumbens* in response to aluminium treatment. Aluminium is known to induce increased organic acid exudation in a number of species (Hue *et al.,* 1986; Bartlett and Riego, 1972; Muchovej, *et al.,* 1988; Ma *et al.*, 1997a; Ma *et al.,* 1998;

Zheng *et al.*, 1998; Ma *et al.*, 1997c; Mugai *et al.*, 2000; Yang *et al.*, 2000; Shuman *et al.*, 1991), which are thought to be involved in phosphate acquisition and uptake in low phosphate conditions by dissolving aluminium phosphate (Gardner *et al.*, 1983; Lipton *et al.*, 1987; Hoffland *et al.*, 1989; Konishi *et al.*, 1985; Jayman and Sivasubramaniam, 1975). Kinraide. (1993) showed that aluminium increased proton extrusion and increased the electrochemical gradient. This could cause increased nutrient uptake. Chapter Four deals further with nutrient uptake. The stimulation of growth could also be due to alleviation of H^+ toxicity by aluminium (Osaki *et al.*, 1997), stimulation of microbial growth on the root surface (Konishi, 1990) or by replacing some of the functions of boron (Konishi, 1992)

The experiments reported in this chapter were only carried out over short time periods, 10 days and 24 hours. Whether 100 or 200 μM aluminium stimulates *Brachiaria decumbens* root growth under long-term (months and years) continual exposure is unclear from these experiments.

Callose induction is stimulated equally by 200 and 400 μM aluminium treatment in *Brachiaria decumbens.* However the root growth data shows 200 μM aluminium to have no effect on root growth. This asks the question whether root growth inhibition or callose induction is the better indicator of aluminium toxicity. Work by other groups have clearly shown good correlation between callose induction and root growth inhibition for indicating both aluminium toxicity at differing aluminium treatments and relative aluminium tolerance between different cultivars and species (Horst *et al.*, 1997; Massot *et al..*, 1999). In this chapter callose concentration agrees with root elongation data in discerning relative aluminium tolerance. However callose induction clearly shows 200 μM aluminium to be toxic to *Brachiaria decumbens* whereas root growth data shows *Brachiaria decumbens* to be completely tolerant to this level of aluminium treatment. So which is the better measure of aluminium toxicity? The induction of callose and decreased root and shoot biomass clearly shows that 200 μM aluminium is causing a physiological response, but is this level of aluminium detrimental to the general health and growth of the plant? In my

opinion growth is generally the better indicator of plant health. Plants are well able to cope with some injury or damage without it causing poor health. Further evidence for answering this question comes in Chapter Four, looking at the effect of aluminium on nutrition.

3.5 Conclusions

Brachiaria decumbens is much more aluminium tolerant than *Brachiaria ruzuziensis*, and both *Brachiaria* species are an order of magnitude more aluminium tolerant than *Triticum aestivum* cv. atlas.

Callose is a more sensitive indicator of aluminium toxicity than inhibition of root elongation. Induction of callose occurs between 2 and 4 hours of aluminium treatment in *Brachiaria decumbens* and *B. ruziziensis,* inhibition of root elongation occurs later, between 4 and 6 hours after start of treatment.

Brachiaria decumbens and *Brachiaria ruzuziensis* show differing allocation of resources to shoots and roots.

Chapter Four: Effect of Aluminium on Plant Nutrition

4.1 Introduction

Soil acidification involves the leaching of macronutrients, such as magnesium, calcium and potassium, from the upper soil horizons and replacement by trivalent aluminium ions and protons (Bolan *et al.,* 1991; Blake *et al.,* 1999). The mobilised aluminium in the soil can further bind soluble phosphate (Clarkson, 1967), inhibit root growth and reduce nutrient uptake (Foy *et al.,* 1978; Foy, 1988; Blamey and Dowling, 1995). Visible symptoms of aluminium stress in leaves of herb and grass species mimic calcium, magnesium, phosphate and iron deficiencies, causing chlorosis and necrosis of leaf tips, smaller leaf size and later maturation (Otsuka, 1969). These problems combine to make low pH soils relatively infertile and less productive. Only plants able to overcome these nutrient limiting conditions will thrive.

4.1.1 Aluminium uptake

Movement of aluminium from the soil solution to the plant root surface is probably mostly due to mass flow of bulk soil solution and to a lesser extent due to diffusion (Marschner, 1995). This is due to the relatively high aluminium concentration in acid soil solutions and the low aluminium uptake by plants, which would be insuffient to provide a large diffusion gradient. Studies have shown that aluminium is able to penetrate the cortex through the apoplastic spaces, however movement into the stele is restricted by the endodermis (Jentschke *et al.*, 1991). However at the root tips, which contain the higher aluminium concentrations (Ryan *et al.*, 1993), there is no endodermis to prevent entry of aluminium into the central root tissue.

The method of movement of aluminium into the symplast is yet unknown (Delhaize and Ryan, 1995), highly charged aluminium ions are unable to diffuse across the lipid bilayer and no channels have yet been found (Delhaize and Ryan, 1995). Methods such as endocytosis and membrane leakage have also been suggested for movement of aluminium uptake into the cell. Despite this hole in our knowledge, it is clear that some aluminium is entering the symplast.

Increasing aluminium treatment has been shown to cause increasing aluminium concentration in root tissue (Vasquez *et al.,* 1999). Mobility of aluminium and transport to above ground vegetation is thought to be relatively low. There is inconclusive evidence for the amount of aluminium entering the cytosol in comparison to apoplastic bound aluminium and the relative importance of aluminium localisation to aluminium toxicity (Delhaize and Ryan, 1995; Taylor, 1995).

In the aluminium accumulator *Melastoma malabathricum* high levels of aluminium is stored in the leaf tissue, particularly the upper epidermis and mesophyll (Watanabe *et al.,* 1998a). Higher concentrations were found in mature rather than young leaves. In the roots aluminium was distributed throughout the roots but concentrated in the epidermis and endodermal tissue (Watanabe *et al.,* 1998a). Forty to sixty-percent of the aluminium was complexed to organic molecules, particularly citrate, which increased in response to aluminium. The remaining aluminium was monomeric aluminium bound to pectins and hemicelluloses (Watanabe *et al.*, 1998a).

Exact measurements of symplastic aluminium have been unsuccessful due to contamination with the apoplastic aluminium or being unable to reliably distinguish symplastic and apoplastic aluminium (Reid *et al.,* 1995; Zhang and Taylor, 1990; Archambault *et al.,* 1996a). Current evidence suggests that most aluminium is concentrated on the root surface and cell walls of epidermal and cortical cells (Mangabeira *et al.,* 1999; Bengtsson, 1992), associated with negative carboxyl and hydroxyl groups of pectin molecules, and as deposits of aluminium phosphate,

aluminium-organic acid complexes or other solid phase aluminium compounds (Mangabeira *et al.,* 1999; Bengtsson, 1992). Estimates of the percentage of tissue aluminium located in the apoplast vary from 50% to 80 or 90%. Recent studies looking at aluminium uptake have used exposure of roots to simple salts to reduce precipitation of solid phase aluminium in the cell wall and membrane surface (Zhang and Taylor, 1990).

It was previously thought that aluminium was unable to enter the symplast except after severe disruption of cell structure or uptake of a neutral aluminium complex (Kochian and Jones, 1996). However, research using techniques such as x-ray microanalysis has shown aluminium to rapidly enter the symplast and accumulate in vacuoles (Vasquez *et al.,* 1999). Aluminium uptake seems to occur in two phases, an initial rapid uptake phase, probably an accumulation in the apoplast (Zhang and Taylor, 1989), followed by a slower linear phase, thought to represent uptake into the symplast (Zhang and Taylor, 1989) but also metabolic binding of aluminium or accumulation of insoluble aluminium precipitates in the apoplast (Zhang and Taylor, 1990; Archambault *et al.,* 1996a)

Aluminium, due to its high reactivity, is thought to be largely unable to move across plasma membranes through cation channels (Gassman and Schroeder, 1994) or by diffusion across the lipid bilayer (Kochian and Jones, 1996). Symplastic aluminium root tip concentrations are thought to be in the region of 5 μM (Reid *et al.,* 1996) to 355 μM (Lazof *et al.,* 1994; Lazof *et al.,* 1996).

Experiments by Archambault *et al.* (1997) have found no difference in total aluminium concentration of mature root compared to root tip tissue. They also found no difference in total aluminium concentration between root tips of sensitive *Triticum aestivum* cultivars ('Scout') compared to tolerant *Triticum aestivum* cultivars ('Atlas'). However removal of exchangeable aluminium bound to apoplastic cation exchange sites and removal of mucilage show that aluminium concentration was higher in root tips and higher in the aluminium sensitive cultivar

(Archambault *et al.*, 1996b). This indicates that tolerant species are better able to exclude aluminium from entering the symplasm.

Further evidence of differentiation of aluminium tolerance based on root tip aluminium concentration can be found in studies by Rincon and Gonzalez (1992), Delhaize *et al.* (1993a), and Samuels *et al.* (1997).

The consensus indicates that aluminium tissue concentrations are generally higher in sensitive and lower in tolerant species (Pintro *et al.*, 1998; Archambault *et al.*, 1998, Samuels *et al.*, 1997), indicating that tolerance is most likely due mainly to an aluminium exclusion mechanism. However some accumulator species such as *Melastoma malabathricum* and *Camellia sinensis* can accumulate extremely high levels of aluminium in shoots and root tissue (Osaki *et al.*, 1997; Lian *et al.*, 1998).

The importance of apoplastic verses symplastic aluminium in causing inhibition of root elongation and other symptoms of aluminium toxicity is unresolved. It has been hypothesised that apoplastic aluminium may change cell wall properties, making the cell wall less elastic and less permeable to water, which would reduce root growth (Horst, 1995). Proponents of apoplastic site of attack point out the low radial mobility of aluminium (Horst, 1995) and its high binding affinity for common cytosolic metabolites such as organic acids and phosphate, which would prevent aluminium easily reaching the nucleus and other vital cell components (Horst, 1995). However it may be that it is when aluminium entering the cell overcomes the cells capacity to safely bind it that aluminium becomes toxic to the cell.

4.1.2 Calcium

Calcium plays a very important role in plants through its effect on calcium modulated proteins and their target molecules (Bush, 1995). Cell metabolism is thought to be closely regulated by changes in calcium concentration within the cell

and across membranes and organelles (Muto, 1992). This regulatory role of calcium is called the calcium homeostat (Bush, 1995).

Signal events are transmitted to the relevant parts of the cell by spikes in calcium concentration (Poovaiah *et al.,* 1993). Different stimuli can cause different spatial and temporal changes in calcium concentration (Gilroy *et al.,* 1993). These spatial calcium gradients may be across membranes, but there may also be calcium concentration gradients within organelles and within areas of the cytoplasm (Gilroy *et al.*, 1991). Calcium signalling can trigger a number of important cell processes including gene expression (Lam *et al.*, 1989; Bowler *et al.*, 1994), mitosis (Lino *et al.*, 1989), cytoskeletal development (Billger *et al.,* 1993; Hepler, 1994), carbohydrate metabolism (Brauer *et al.*, 1990), vesicle fusion, motility (Williamson and Monck, 1989), ionic balance (Ketchum and Poole, 1991) and secretion (Zorec and Tester, 1992). Calcium is also required by a number of enzymes and proteins, such as phospholipase C (Schmidt *et al.,* 2001). Any stimuli which cause a complex cell response are likely to involve the calcium homeostat at some point (Bush, 1995).

Control of cytosolic calcium concentration is through the activity of calcium pumps and channels (Evans *et al.,* 1991; Johannes *et al.,* 1991). Efflux from the cytosol is via the Ca-ATPases and calcium/proton antiporters, and uptake by membrane calcium channels (Bush, 1995). Environmental factors which affect calcium uptake can therefore have a potentially considerable effect on cell metabolism.

It has been suggested that aluminium may inhibit root growth through its effect on the calcium homeostat and calcium signal transduction, which controls both cell division and cell elongation (Kochian, 1995; Rengel, 1992; Taylor, 1990). Aluminium has been shown to block calcium channels (Huang *et al.,* 1996; Rengel and Elliot, 1992) and affect cytoplasm calcium concentration in a number of species. In *Oryza sativa* (rice) cultivars, treatment with increasing aluminium concentrations caused a decrease in root calcium content in the sensitive cultivar but had no effect on root calcium concentrations in the tolerant and intermediate cultivar (Jan, 1991).

In the same experiment, shoot calcium concentrations increased in the aluminium tolerant cultivar and decreased in the sensitive cultivar in response to increasing aluminium treatment.

Similar effects on calcium uptake have been shown in *Triticum aestivum* (wheat) cultivars (Ohki, 1985), with aluminium treatment causing decreased root and shoot calcium concentrations. Huang *et al.* (1992) showed that calcium uptake was decreased by aluminium in sensitive *Triticum aestivum* cultivars but not in tolerant cultivars, and suggested this was due to aluminium directly blocking calcium channels.

In *Fagus sylvatica* (beech) treatment with 1 mM aluminium caused a decrease in calcium uptake (217 to 63 μmol/g) and transport to shoots (Asp *et al.*, 1991). In an aluminium accumulating plant, *Melastoma malabathricum*, aluminium treatment had no effect on root calcium concentration (1.3 mg/g and 1.4mg/g) and increased shoot calcium content (2.7 and 4.3 mg/g) (Watanabe *et al.*, 1997). In aluminium sensitive plants (*Hordeum vulgare*), root and shoot calcium concentration were both decreased by aluminium treatment (roots 2.8 to 0.8 mg/g; shoots 3.5 to 1.9 mg/g) (Watanabe *et al.*, 1997).

Aluminium-induced growth inhibition and decreased calcium uptake has been shown to be ameliorated by increasing the concentration of calcium in the hydroponic nutrient solution (Kinraide, 1998). Three mechanisms for this amelioration were proposed; a) displacement of cell surface bound aluminium by calcium inducing a reduction in the negative charge of the cell surface. b) replacement of aluminium by calcium at cell surface therefore preventing calcium limiting conditions. c) Aluminium/calcium interactions at the cell surface (Kinraide, 1998).

One proposed mechanism of aluminium toxicity is by the replacement of calcium with aluminium in the cell wall and apoplastic binding sites (Rengel, 1992). This would affect both cell wall and membrane structure and properties and also decrease

the calcium available for uptake into the cytosol to maintain cell calcium levels and fluxes (Rengel, 1990; Huang *et al.,* 1992). However work by Schofield *et al.* (1998) has indicated that aluminium replaces only a fraction of the calcium bound to apoplastic structures. If aluminium toxicity is partly due to an effect on calcium metabolism then it must therefore be by another mechanism such as blocking of calcium channels (Schofield *et al.*, 1998).

Evidence for blocking of calcium channels by aluminium is provided by work on BY-2 *Nicotiana tabacum* cell cultures (Jones *et al.,* 1998a). Aluminium was found to cause a reduction in cytoplasmic calcium concentrations (256 to 64 nM) and inhibit growth. La^{2+} (a known calcium channel blocker) also inhibited growth and decreased cytosolic calcium levels, however unlike aluminium, inhibition was reversed with removal of La^{2+} (Jones *et al.,* 1998a). This indicates that aluminium is toxic in more ways than its effect on calcium metabolism (Jones *et al.,* 1998a). The similarities between the effect of aluminium and La^{2+}, suggest that aluminium acts by blocking calcium channels. External calcium concentrations are very important for maintaining cytosolic calcium concentrations (Jones *et al.,* 1998a).

Enthusiasm for linking aluminium induced growth inhibition and aluminium induced decrease in calcium uptake should be tempered by findings of Jones *et al.* (1998b). Working on *Arabidopsis* root hairs they found that aluminium caused an increase in calcium concentrations in sensitive and wildtype strains but not in tolerant plants. However this change in cytosolic calcium concentration occurred after growth inhibition.

4.1.3 Magnesium

Magnesium and calcium are very similar chemically and compete for cell uptake (Marschner, 1986; Ericsson *et al.,* 1998). Several experiments have shown antagonism between calcium and magnesium for plant uptake due to competition for the same cation channels, with increased apoplastic magnesium concentrations

decreasing calcium uptake and visa versa (DeSousa *et al.*, 1998; Cernohorska *et al.*, 1996; Ericsson *et al.*, 1998; van Oene, 1998).

The effect of aluminium on magnesium nutrition is very similar to its effect on calcium nutrition, generally causing a decrease in cation uptake, probably through blockage of cation channels (Rengel and Robinson, 1989; MacDiarmid and Gardner, 1996; Jan, 1991; Bernal and Clark, 1997). Aluminium has been shown to decrease cell magnesium concentrations in yeast (MacDiarmid and Gardner, 1996). This was thought to be at least in part due to the blocking of cation channels, evidence for which comes from a yeast strain with a defect in cation uptake, which was found to be more sensitive to aluminium.

In three *Oryza sativa* cultivars, aluminium reduced magnesium levels in both roots and shoots irrespective of cultivar sensitivity (Jan, 1991). In the aluminium accumulator *Melastoma. malabathricum*, aluminium treatment caused a decrease in shoot magnesium levels but a small increase in root magnesium levels (Watanabe *et al.*, 1997). In contradiction, a study looking at a range of sensitive and tolerant species found that magnesium root and shoot content was decreased by aluminium treatment irrespective of aluminium sensitivity (Bernal and Clark, 1997). However magnesium root concentrations were generally lower in the 'tolerant' species than the 'sensitive' species.

In *Picea abies* (Norway spruce), magnesium uptake to needles was inhibited by aluminium treatment at lower pH treatments but not at pH 5.0 (Godbold and Jentschke, 1998). Magnesium uptake was found to be unrelated to magnesium concentration at apoplastic cation exchange sites. Under aluminium treatment at pH 5.0 magnesium content of needles was unaffected, however apoplastic Mg was reduced. It was suggested that at pH 5.0 the toxic aluminium species was Al_{13}, which has no effect on magnesium uptake, but at lower pH trivalent aluminium dominated which directly interferes with magnesium uptake (Godbold and Jentschke, 1998).

4.1.4 Potassium

Potassium is important as a counter ion (Marshner, 1995). In studies on a number of species such as *Ilex aquifolium* (holly) (Lasseigne *et al.*, 1997), *Sorghum vulgare* (sorghum) (Bernal and Clark, 1997) and *Zea mays* (Pintro *et al.*, 1998) aluminium treatment was found to decrease potassium root and shoot content. In a study on a range of aluminium sensitive and tolerant species potassium tissue concentration was decreased, however tolerant species were found to have a higher root potassium concentration than more sensitive species and cultivars (Bernal and Clark, 1997). This decrease in potassium uptake is probably due to the direct effect of aluminium on plasma membrane transport, where it has been shown to block potassium ion channels (Huang *et al,* 1996; Gassman and Schroeder, 1994). Not only has aluminium been shown to inhibit potassium uptake (Miyasaka *et al.,* 1989), but aluminium toxicity has also been shown to increase potassium efflux (Wagatsuma *et al.,* 1995).

4.1.5 Iron

Iron is very insoluble in soil solution and resides mainly in its insoluble ferric (Fe^{3+}) phyto-unavailable form (Hughes *et al.,* 1992). It has been estimated that the available soluble ferrous iron (Fe^{2+}) accounts for only 1% of a plants need in soils at pH 4.0, and the solubility of Fe^{2+} decreases a thousand fold with each increase of 1 pH unit (Hughes *et al.,* 1992). However plants have developed mechanisms to maximize uptake of the sparsely available iron. These mechanisms can be classified into two broad types (Romheld, 1987). The first (Strategy I), used by most dicotyledonous plants and most monocots except grasses, involves the exudation of reducing agents or protons and an increase in the activity of iron reductase at the plasma membrane and uses a Fe^{2+} transporter protein across the membrane (Romheld, 1987; Jolley *et al.,* 1996; Hughes *et al,* 1992). The second mechanism (Strategy II) involves the exudation of a group of phytosiderophores (mugineic acids), which bind to iron (Fe^{3+}), solublise the ferric iron and transport across the membrane by an Fe^{3+}-Phytosiderophore complex transporter (Ma and Nomato, 1996).

Aluminium has been shown to inhibit iron nutrition and cause iron deficiency in a number of species including *Triticum aestivum* (Chang *et al.*, 1998), *Ilex aquifolium* (Lasseigne *et al.*, 1997), *Zea mays*, where shoot content but not root concentration decreased (Pintro *et al.*, 1998) and *Sorghum vulgare* (Bernal and Clark, 1997). A study by Chang *et al* (1998) found *Triticum aestivum* supplied with insoluble ferric iron hydroxide as its iron source suffered iron deficiency with aluminium treatment but not in control plants. Looking at the synthesis and exudation of phytosiderophores, aluminium was found to inhibit both synthesis and exudation within 3 hours of aluminium treatment (Chang *et al.*, 1998).

Another study by Chang *et al.,* (1999a) looking at the effect of aluminium on phytosiderophore iron solublisation capacity, iron binding and Fe^{3+} phytosiderophore complex uptake, showed that there was no effect on iron binding or solublisation capacity of phytosiderophore DMU, indicating that aluminium has no direct effect on binding. Aluminium also had no effect on uptake over short periods of aluminium exposure, however longer (greater than six hours) periods which also caused decreased elongation, caused a 50% decrease in iron-phytosiderophore uptake (Chang *et al.,* 1999a). This decrease in uptake was thought to be due to an aluminium effect on general cell metabolism and not directly on iron uptake (Chang *et al.,* 1999a).

Iron has also been implicated in aluminium toxicity, involved in causing increased lipid peroxidation when associated with aluminium treatment (Yamamoto *et al.*, 1997; Ikegawa *et al.*, 1998).

4.1.6 Phosphate

Phosphate is probably the most limiting nutrient for productivity and yield in *Brachiaria* pastures (Sanchez and Salinas, 1981; Salinas *et al.,* 1990). It is involved in energy transfer, metabolic regulation and control and as a building block for a

wide range of cell metabolites and structural molecules (Duff *et al.,* 1994). Phosphate is preferentially taken up by plants in its orthophosphate form (Greaves *et al.,* 1963; Greaves *et al.,* 1967). Orthophosphate is readily bound by aluminium and iron to form insoluble aluminium and iron phosphates. Thirty to ninety percent of soil phosphate can be in organic form, which is less available for uptake by plants (Dalal, 1978; Pederson, 1953). Plants adapted to low phosphate soils, such as acid soils, have developed mechanisms for increasing phosphate uptake, including exudation of organic acids and phytases (Duff *et al.*, 1994) and associations with mycorrhiza and bacteria (Akiyama *et al.,* 2002; Olsson *et al.,* 2002). Despite the effect of aluminium on the availability of phosphate, most studies, mainly involving hydroponic culture, have found root phosphate concentrations to increase with aluminium treatment (Pintro *et al.,* 1998; Mukherjee and Asanuma, 1998; Lasseigne *et al.*, 1997).

Work on *Triticum aestivum* and microbes such as *Bradyrhizobium japonicum* have shown aluminium to stimulate increases in cellular orthophosphate concentrations and increase phosphate exudation (Mukherjee and Asanuma, 1998). Increased cellular and extracellular phosphate are thought to act as possible aluminium defence mechanisms, binding aluminium and protons in soil solution, and either preventing aluminium entry to cells or safely binding aluminium already in the cell (Pellet *et al.,* 1997). Aluminium tolerant strains of *Bradyrhizobium japonicum* are able to accumulate and secrete higher concentrations of inorganic phosphate than more sensitive strains (Mukherjee and Asanuma, 1998).

4.1.7 Other nutrients

Aluminium has also been shown to reduce boron, copper, zinc and manganese leaf content in *Ilex aquifolium* (Lasseigne *et al.*, 1997) and decrease manganese in *Zea mays* (Pintro *et al.*, 1998). A study on a range of plant species grouped into "aluminium sensitive", "intermediate" and "aluminium tolerant" found that leaf and root concentrations of copper, zinc and manganese were generally increasingly reduced by increasing aluminium treatment (Bernal and Clark, 1997). They also

found that manganese shoot content was higher and root concentration lower in aluminium tolerant species than aluminium sensitive species. In contrast, aluminium was found to increase concentrations of copper and boron in roots and copper in the shoots in sensitive and tolerant *Zea mays* cultivars (Pintro *et al.*, 1998).

In two tomato cultivars uptake of molybdate, copper, zinc, manganese and boron was reduced (Simon *et al.*, 1994). Copper and Molybdate root content increased with aluminium treatment but shoot copper, boron, zinc and manganese concentrations were decreased.

4.1.8 Chapter aims

This chapter investigates the effect of nutrition on aluminium tolerance and the effect of aluminium on the nutrient status of the two *Brachiaria* species in comparison to wheat after exposure to increasing concentrations of aluminium.

4.2 Methods

4.2.1 Experiment One: Effect of nutrition on aluminium tolerance

21-day old *Brachiaria decumbens* and *Brachiaria ruziziensis* plants were treated with low or high strength nutrient solution (Table 4.1) with or without 100 μM aluminium chloride. Root growth and root and shoot biomass measurements were taken as described in Chapter 3. Sub-samples of tissue were taken, wet ashed and analysed for aluminium, calcium, magnesium, potassium, iron and zinc concentration (see below for method). Five pot replicates were used for each treatment.

Use of the computer software 'Geochem' (Parker *et al.*, 1987; Parker *et al.*, 1995) predicts that the free soluble aluminium concentration in the two solutions will be 70 and 56 μM in the low and high nutrient solutions respectively.

Table 4.1: Concentration of nutrient treatments

Nutrient	*Low nutrient solution, μM*	*High nutrient solution, μM*
NH_4NO_3	75	900
Na_2SO_4	20	100
K_2SO_4	75	300
$MgSO_4$	50	300
$CaCl_2$	75	400
KH_2PO_4	3	90
$MnSO_4$	1.25	15
$FeCl_3$	1.25	15
$NaMoO_4$	0.01	0.5
$ZnSO_4$	0.01	0.5
$CuSO_4$	0.01	0.5
H_3BO_3	0.05	5

4.2.1.1 Tissue cation measurements

The plants were harvested, dried in an oven at 100°C for 5 days, then a sub-sample of root and shoot tissue taken. The samples were wet ashed by weighing approximately 0.5 g dried root or shoot into Teflon combustion chambers, 1.5 ml fuming nitric acid was added, the pots sealed and heated to 160°C for 10 hours. The digested sample was filtered and made up to 25 ml with distilled water (E-Pure,

Barnstead). Cation analysis was carried out using an inductively coupled plasma mass spectrometer. The results are shown as μg cation per mg dry weight of sample.

4.2.2 Experiment Two: Effect of aluminium dose on tissue nutrient concentrations

Fourteen-day old plants were treated under standard growth conditions (Chapter Two) with 0, 200, 400 or 800 μM aluminium chloride for 24 hours, then harvested, wet-ashed and analysed for cation content (see section 4.2.1.1. for method). Five pot replicates were made for each treatment.

Phosphate tissue concentrations were measured during the organic acids dose response experiment. 21-day old *Brachiaria decumbens, Brachiaria ruziziensis* and *Triticum aestivum* plants were treated with 0, 50, 100, 200, 400 or 800 μM aluminium under standard hydroponic growth conditions described in Chapter Two. Phosphate root tip concentration was measured by capillary electrophoresis. Experimental methods are described in Chapter Two for organic acids and anions.

4.3 Results

4.3.1 Experiment One: Effect of nutrition on aluminium tolerance

4.3.1.1 Root Growth

Under high nutrient conditions 100 μM aluminium had no significant effect on *Brachiaria ruziziensis* root growth, whereas under low nutrient conditions there was a decrease in *Brachiaria ruziziensis* root growth after treatment with 100 μM aluminium (Figure 4.1). Due to the high variability of the root length measurements the difference is not statistically significant (2-sample t-test P=0.095). The more

resistant species, *Brachiaria. decumbens*, showed no effect of 100 μM aluminium on root growth at either low or high nutrient levels (Figure 4.1).

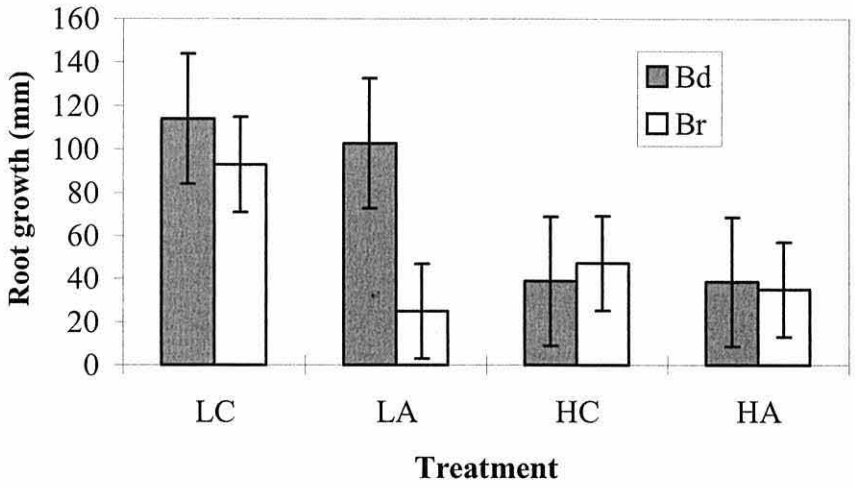

Figure 4.1: *Brachiaria decumbens* and *Brachiaria ruziziensis* root growth (of the longest root) after treatment for ten days. L = low nutrient conditions, H= high nutrient conditions, C = 0 μM Al treatment and A = 100 μM Al. ± S.E. n = 5.

4.3.1.2 Root Biomass

Brachiaria ruziziensis root biomass increased with treatment with higher nutrient levels (p=0.006), but *Brachiaria decumbens* was unaffected by nutrient level (Figure 4.2). Treatment with aluminium had no effect on *Brachiaria ruziziensis* root biomass at either high or low nutrient treatments, however increased *Brachiaria decumbens* root biomass under high nutrient conditions but not low nutrient conditions (Figure 4.2). This increased root biomass in *Brachiaria decumbens* was not reflected in the root length data, which showed no difference between aluminium and control treatments. This indicates that even in *Brachiaria decumbens,* which had unaffected root growth after aluminium treatment, aluminium is still causing a

change in root morphology and structure. The increase in response to aluminium could be due to the effect of aluminium on availability of key nutrients such as P, stimulating the root to allocate more biomass to the roots to compensate.

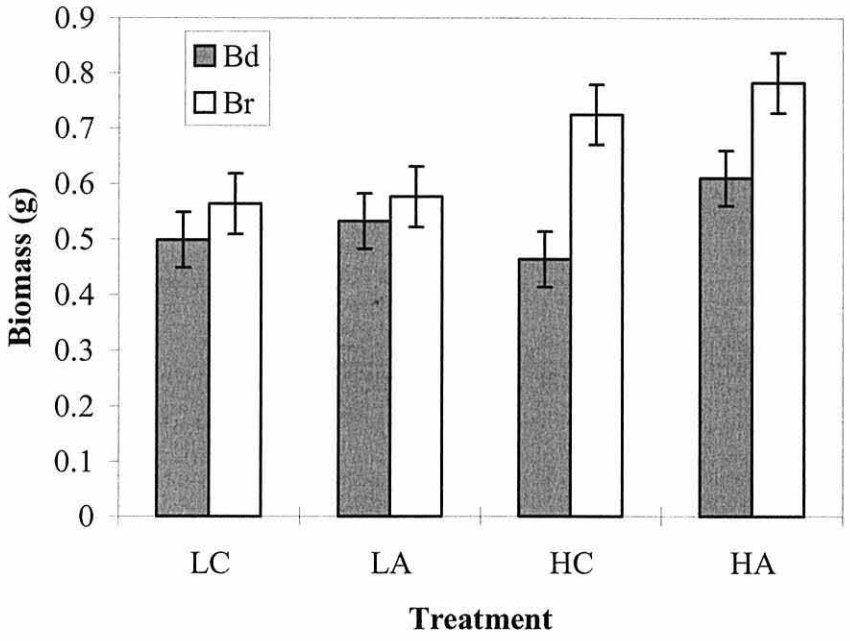

Figure 4.2: *Brachiaria decumbens* and *Brachiaria ruziziensis* root biomass after treatment for ten days. L = low nutrient conditions, H= high nutrient conditions, C = 0 μM Al treatment and A = 100 μM Al. ± S.E. n = 5.

4.3.1.3 Shoot Biomass

Nutrient treatment had a huge effect on shoot biomass, with much higher shoot biomass in the high nutrient treatment for both *Brachiaria decumbens* ($p=0.002$) and *Brachiaria ruziziensis* ($p<0.001$) (Figure 4.3). Aluminium had no effect on shoot biomass in either species. There was a trend for higher shoot biomass in *Brachiaria decumbens* than *Brachiaria ruziziensis* under all treatment conditions, how. ($p=0.036$ at high nutrient level and $p=0.062$ at low).

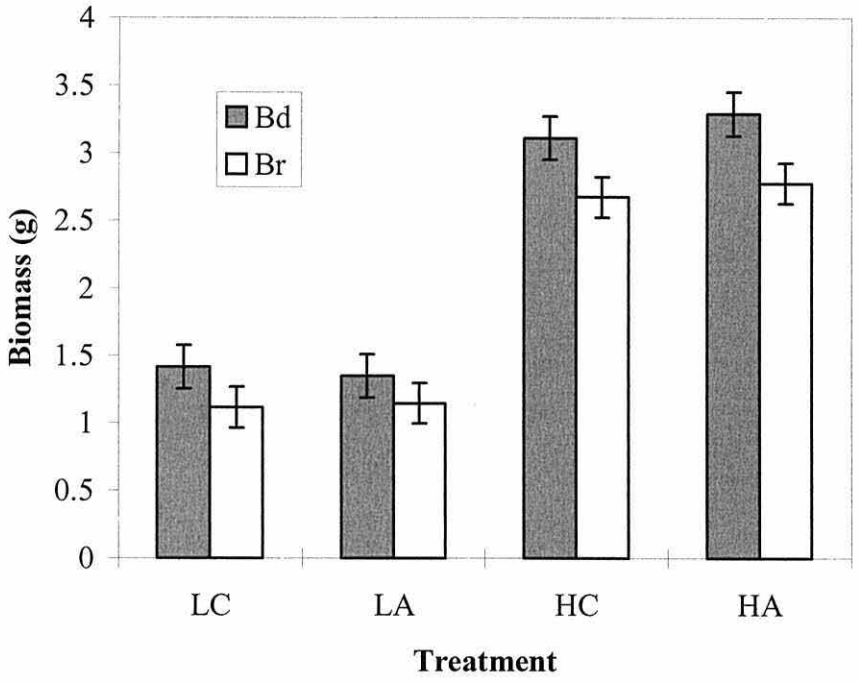

Figure 4.3: *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) shoot biomass after treatment for ten days. L = low nutrient conditions, H = high nutrient conditions, C = 0 μM Al treatment and A = 100 μM Al. ± S.E. n = 5.

4.3.1.4 Root:Shoot Ratio

Root:shoot ratio was much higher in *Brachiaria ruziziensis* than *Brachiaria decumbens* under all treatment conditions (LC $p=0.015$; LA $p=0.026$; HC $p<0.001$; HA $p<0.001$) (Figure 4.4). Root:shoot ratios were higher for both species in the low nutrient conditions than the high nutrient conditions ($p=0.002$). Aluminium had no statistically significant effect on root:shoot ratio ($p>0.05$), however there was a trend in both species and under high and low nutrient conditions for an increase in root:shoot ratio with aluminium treatment.

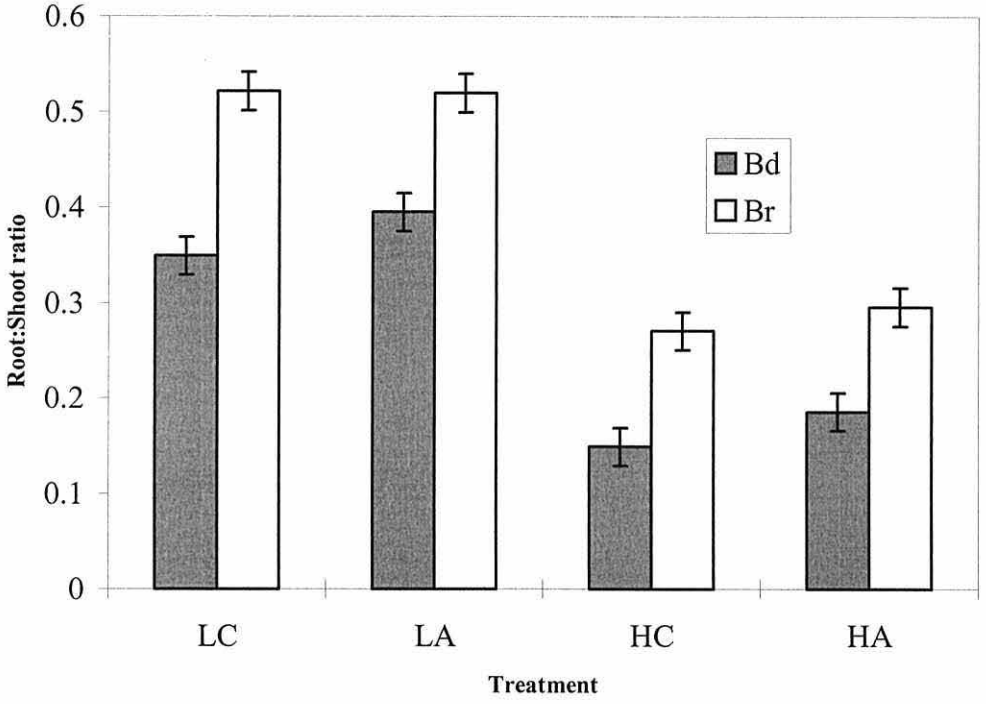

Figure 4.4: *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) root:shoot ratio after treatment for ten days. L = low nutrient conditions, H= high nutrient conditions, C = 0 μM Al treatment and A = 100 μM Al. ± S.E. n = 5.

4.3.1.5 Aluminium tissue concentrations

Brachiaria decumbens and *Brachiaria ruziziensis* aluminium root tissue concentrations were not significantly different after aluminium treatment (Figure 4.5). There was also no effect of nutrient treatment on aluminium root content in either *Brachiaria* species.

Aluminium shoot concentrations followed the same pattern as root concentrations. There were no species differences in aluminium shoot concentration at either high ($p=0.397$) or low nutrient treatment ($p=0.363$) (Figure 4.6). There was also no nutrient effect on aluminium shoot content in either species (Bd $p=0.44$, Br $p=0.534$). The aluminium tissue content was much lower in shoots than roots of both species.

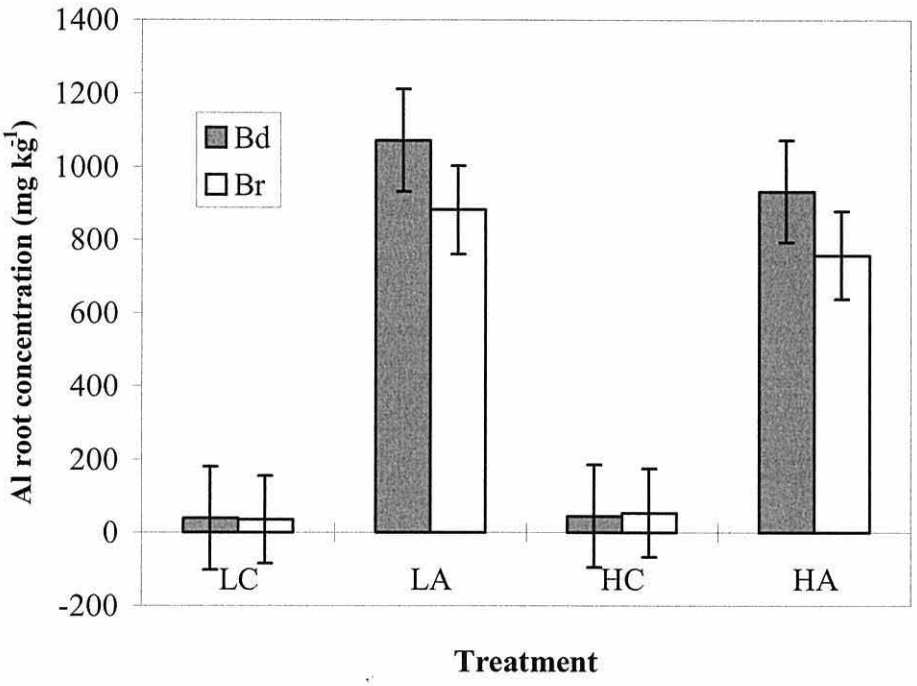

Figure 4.5: Root aluminium concentrations (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* and *Brachiaria ruziziensis* at high (H) and low (L) nutrient concentrations with 0 (C) or 100μM (Al) aluminium. ± S.E. n = 5.

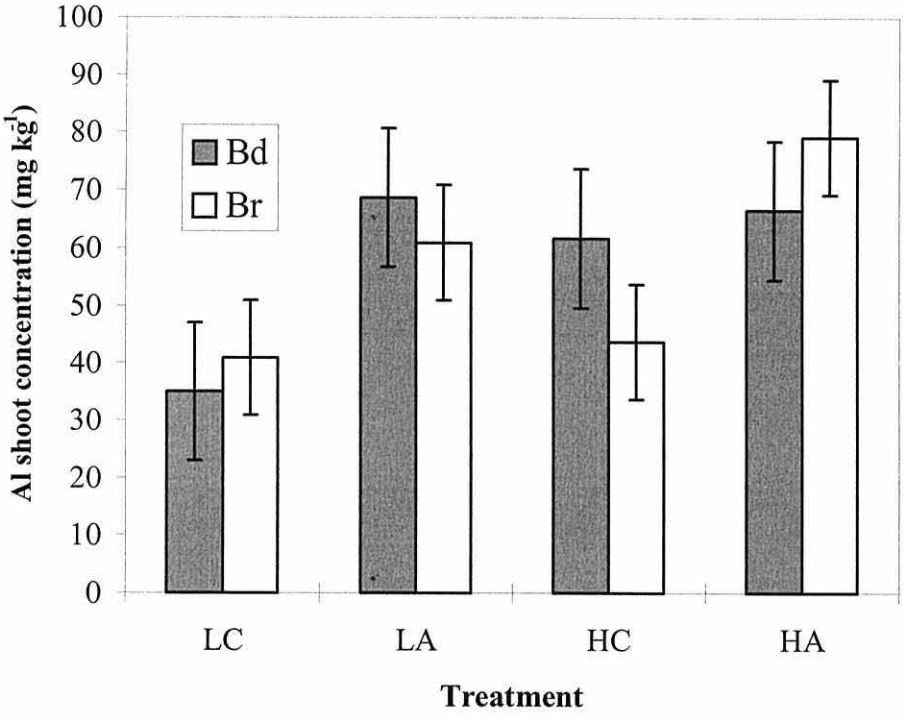

Figure 4.6: Aluminium shoot concentrations (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100μM (Al) aluminium. ± S.E. n = 5.

4.3.1.6 Tissue cation content

There were no species differences in calcium root concentration (Figure 4.7). Aluminium treatment had no effect on calcium root concentration under either low or high nutrient conditions in either *Brachiaria* species (Figure 4.7). Treatment with higher nutrient concentrations increased calcium root concentrations in both species ($p<0.001$).

Calcium shoot concentrations were unaffected by either aluminium treatment or different nutrient treatment (Figure 4.8). There was also no difference in calcium shoot concentration between the two *Brachiaria* species.

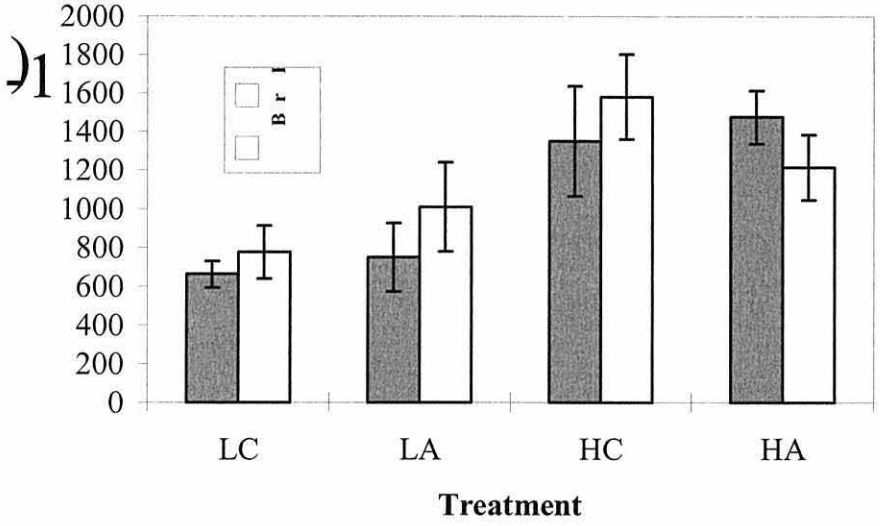

Figure 4.7: Calcium root concentrations (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100 µM (Al) aluminium. ± S.E. n = 5.

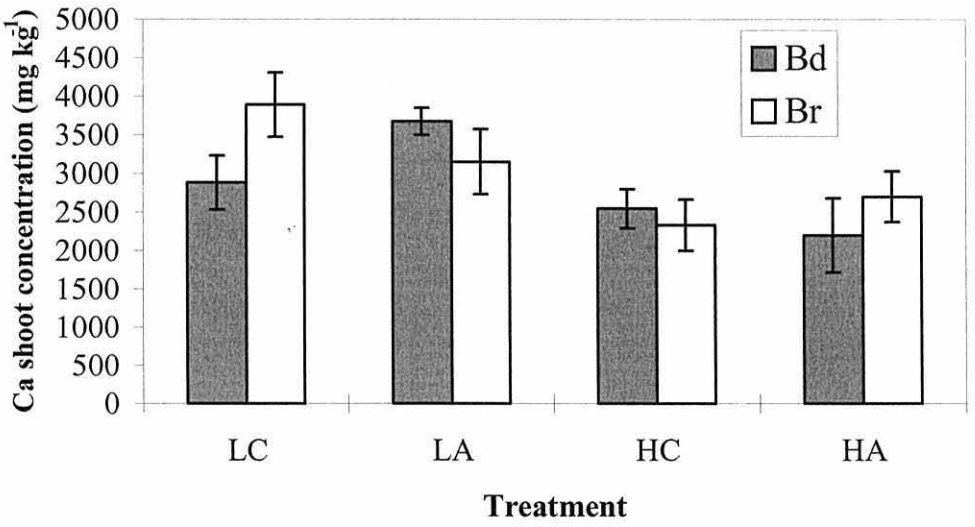

Figure 4.8: Calcium shoot concentrations (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100 µM (Al) aluminium. ± S.E. n = 5.

Aluminium treatment caused a significant increase in root magnesium concentration in both *Brachiaria* species at low nutrient conditions (Bd p=0.059, Br p=0.033), but caused a decrease under high nutrient conditions (Bd p=0.104, Br p=0.053) (Figure 4.9). There was no difference between *Brachiaria decumbens* and *Brachiaria ruziziensis* in their root magnesium concentrations. *Brachiaria decumbens* magnesium shoot concentration was unaffected by aluminium treatment at both high and low nutrient treatment (Figure 4.10). *Brachiaria ruziziensis* magnesium shoot content was decreased by treatment with aluminium at low nutrient conditions (p=0.09) but not at high nutrient conditions (Figure 4.10).

Nutrient treatment had no effect on magnesium shoot concentration in *Brachiaria decumbens,* but treatment with high nutrient solution caused a significant increase in shoot magnesium content in *Brachiaria ruziziensis* (p=0.033) (Figure 4.10).

Potassium root content was unaffected by either aluminium or nutrient treatment in either *Brachiaria* species (Figure 4.11). There was also no difference between the two *Brachiaria* species.

Aluminium had no effect on shoot potassium concentrations of either species under high or low nutrient conditions (Figure 4.12). However there was an increase in shoot potassium levels in *Brachiaria ruziziensis* under high nutrient treatment in comparison to the low nutrient treatment (p=0.037) (Figure 4.12)

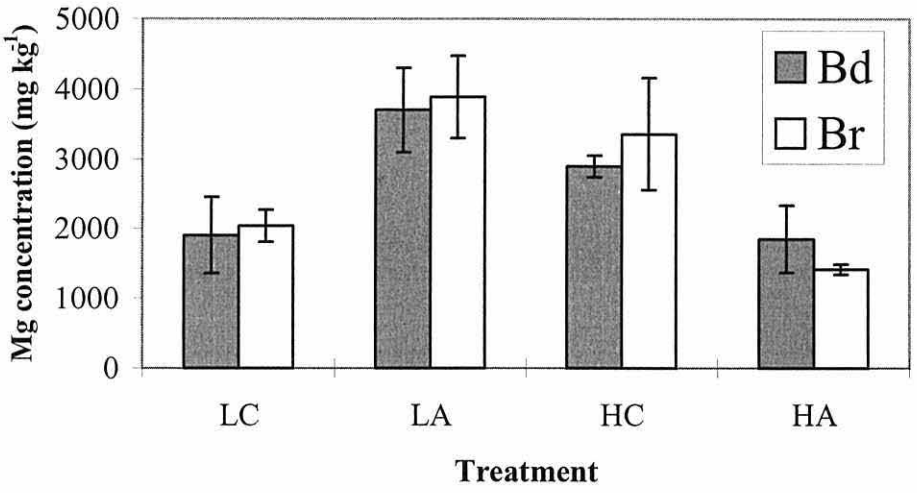

Figure 4.9: Magnesium root concentrations (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100 µM (Al) aluminium. ± S.E. n = 5.

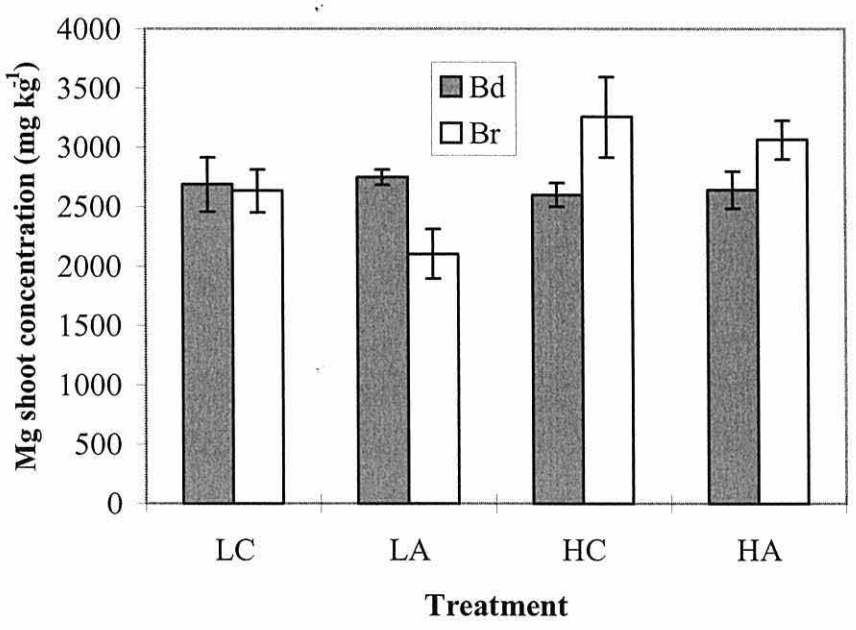

Figure 4.10: Magnesium shoot concentrations (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100 µM (Al) aluminium. ± S.E. n = 5.

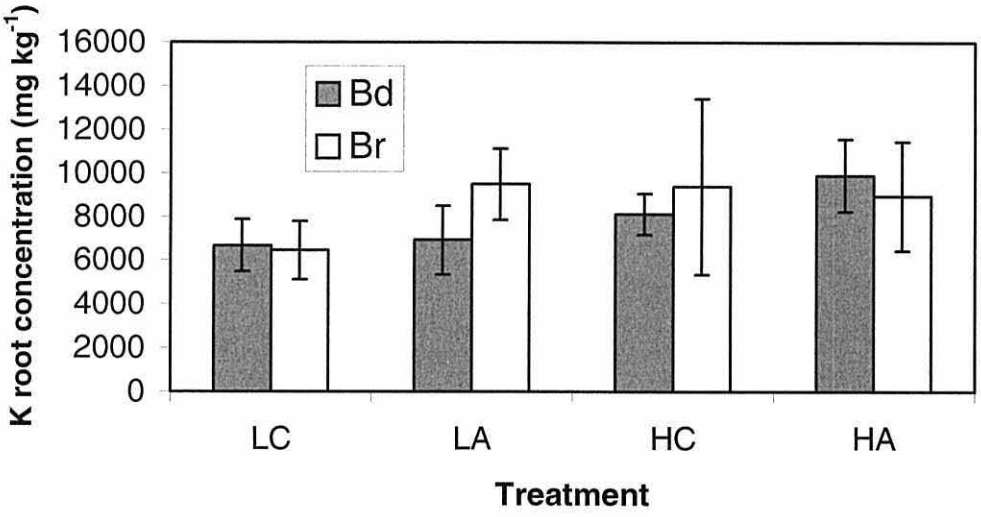

Figure 4.11: Potassium root concentrations (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100 µM (Al) aluminium. ± S.E. n = 5.

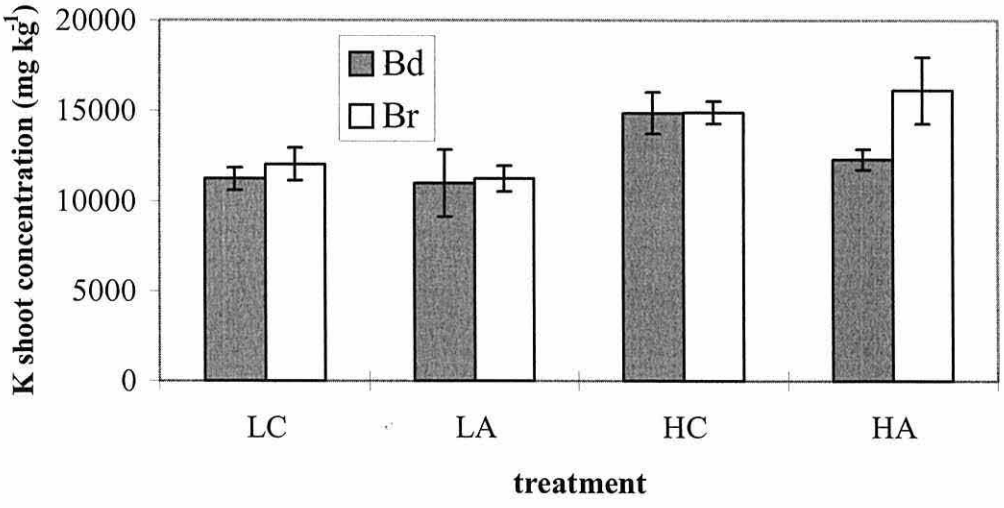

Figure 4.12: Potassium shoot concentrations (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100 µM (Al) aluminium. ± S.E. n = 5.

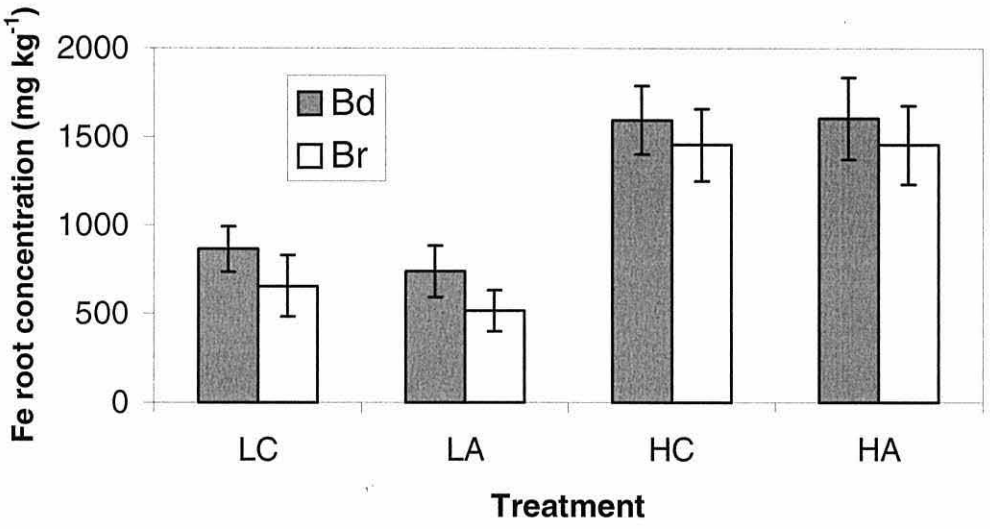

Figure 4.13: Iron root concentrations (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100 μM (Al) aluminium. ± S.E. n = 5.

There were no differences in iron root concentration between the two *Brachiaria* species (p=0.136) (Figure 4.13). Iron root concentration was unaffected by aluminium treatment in either *Brachiaria* species under low or high nutrient conditions (Figure 4.13). Root iron concentration was increased by treatment with high nutrient conditions in both species (Bd and Br $p<0.001$).

Shoot iron concentrations were higher under high nutrient conditions in both species (p=0.004) (Figure 4.14). Aluminium had no effect on shoot iron concentration in *Brachiaria decumbens* but increased iron content in *Brachiaria ruziziensis* under both nutrient conditions (p=0.104). *Brachiaria decumbens* had significantly higher iron root tip concentration under low treatment (p=0.020).

Aluminium caused an increase in manganese root concentration in *Brachiaria ruziziensis* at low nutrient conditions (p=0.033) and a decrease in manganese concentration under high nutrient conditions (p=0.003) (Figure 4.15). *Brachiaria decumbens* manganese root concentration was unaffected by aluminium under low nutrient conditions but decreased by aluminium under high nutrient treatment (p=0.004) (Figure 4.15).

Manganese shoot concentration was unaffected by either aluminium treatment or nutrient conditions in either *Brachiaria* species (Figure 4.16).

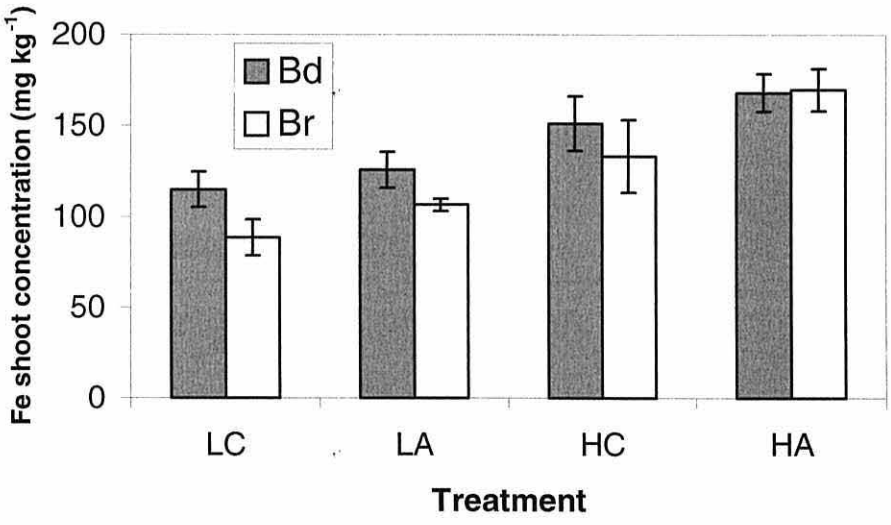

Figure 4.14: Iron shoot concentrations (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100µM (Al) aluminium. ± S.E. n = 5.

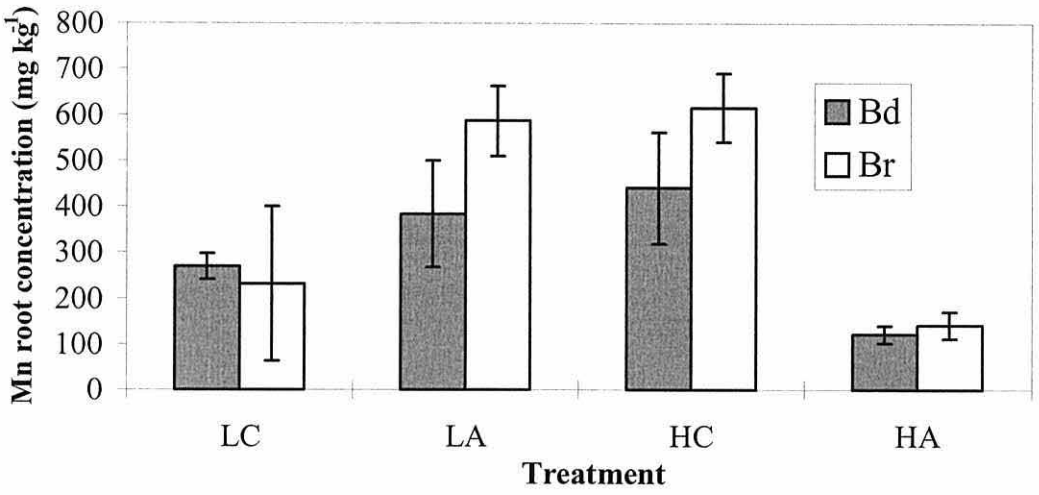

Figure 4.15: Manganese root concentration (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100 µM (Al) aluminium. ± S.E. n = 5.

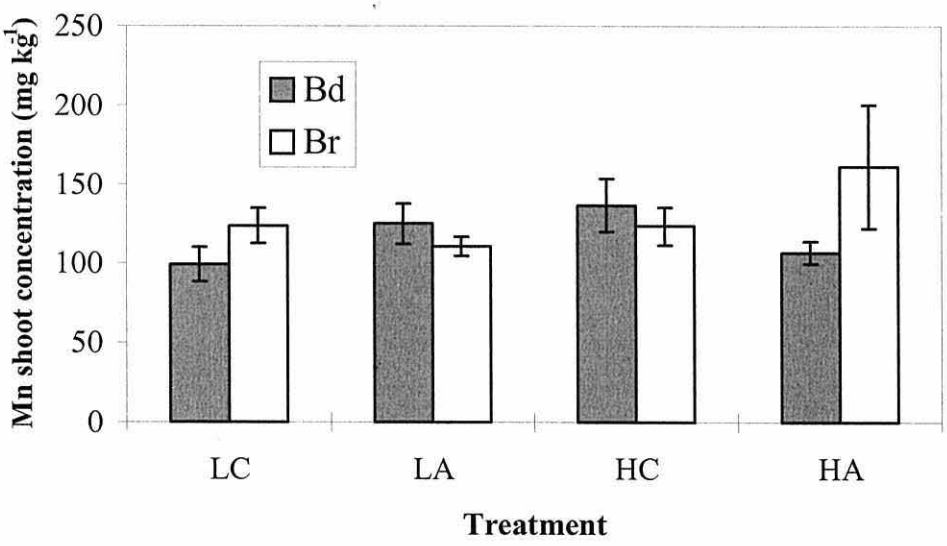

Figure 4.16: Manganese shoot concentration (mg kg^{-1}) in dried root samples *of Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) at high (H) and low (L) nutrient concentrations with 0 (C) or 100 µM (Al) aluminium. ± S.E. n = 5

4.3.2 Experiment Two: Effect of aluminium dose on tissue nutrient concentrations

The following results look at the effect of increasing aluminium treatment on root and shoot cation concentration.

4.3.2.1 Aluminium tissue concentration

Aluminium tissue content increased steadily with increasing aluminium treatment in *Triticum aestivum* (Figure 4.17). There were similar aluminium levels in roots of both *Brachiaria* species after treatment with 200 μM aluminium, the concentration in both *Brachiaria* species being higher than in *Triticum aestivum* at the 200 μM aluminium treatment. Aluminium root concentration was further increased in *Brachiaria ruziziensis* with treatment with 400 μM aluminium, however the levels in *Brachiaria decumbens* remained the same as with the 200 μM aluminium treatment (Figure 4.17). Aluminium levels in *Brachiaria decumbens* increased to the levels found in both *Brachiaria ruziziensis* and *Triticum aestivum* after treatment with 800 μM aluminium. Aluminium root concentrations in the sensitive *Triticum aestivum* never exceeded levels found in either *Brachiaria* species, and the root aluminium concentration was not significantly different between the 3 species ($p=0.435$)

Aluminium shoot concentration gradually increased in both *Brachiaria* species with no difference between *Brachiaria decumbens* and *Brachiaria ruziziensis* (Figure 4.18). The aluminium concentration in shoots of *Triticum aestivum* were more than double and significantly higher than the levels found in either *Brachiaria* species ($p<0.001$).

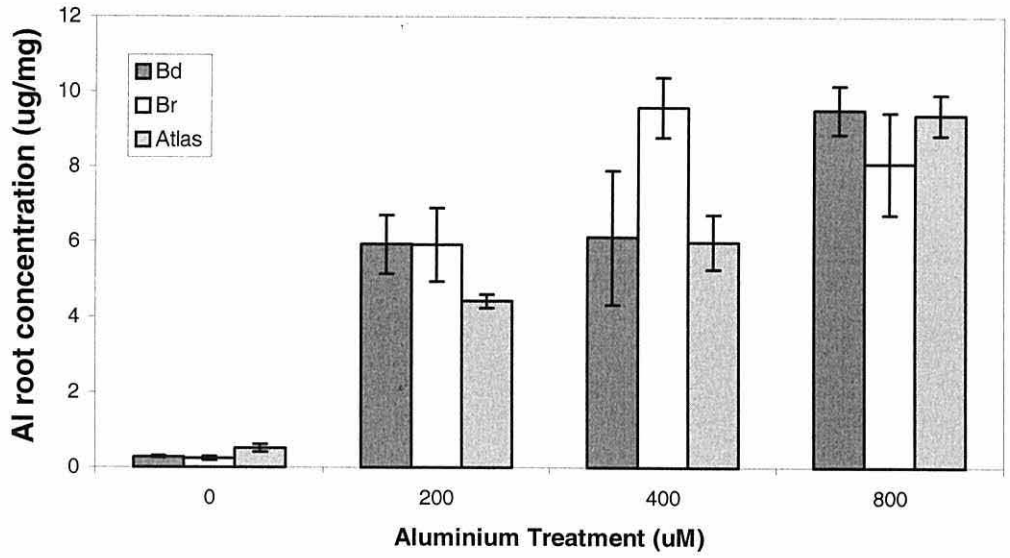

Figure 4.17: Aluminium root tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5

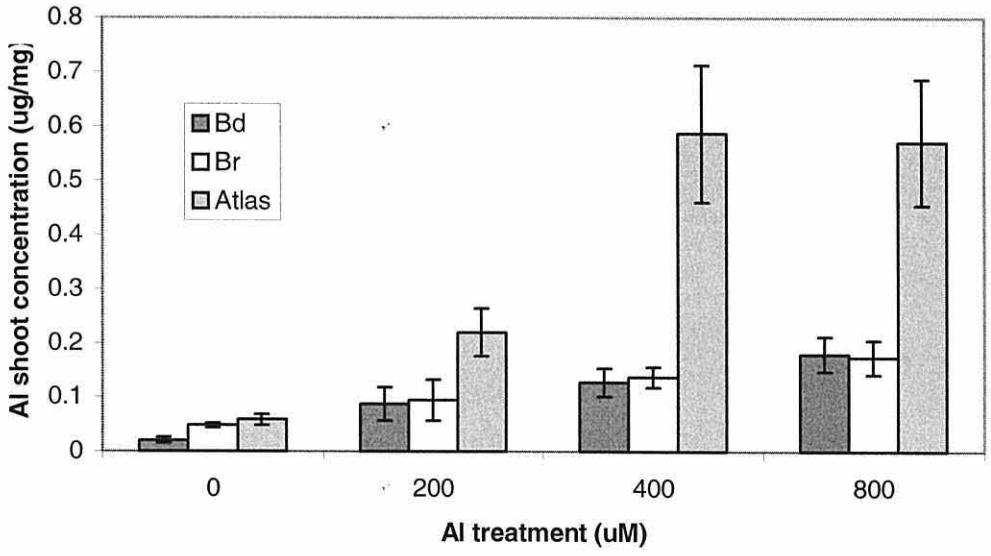

Figure 4.18: Aluminium shoot tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5

4.3.2.2 Tissue Cation Concentration

Calcium root concentration was unaffected by increasing aluminium treatment in all 3 species (Figure 4.19). Aluminium treatment caused a decrease in calcium shoot concentration in all three species (Bd $p=0.009$, Br and Atlas $p<0.001$), however the decrease was bigger in the two *Brachiaria* species than *Triticum aestivum* (Figure 4.20). There was no difference in calcium shoot concentration between the two *Brachiaria* species, but shoot calcium levels were higher in the *Brachiaria* species than in *Triticum aestivum* under control and 200 μM aluminium treatments ($p=0.033$ and 0.005 respectively).

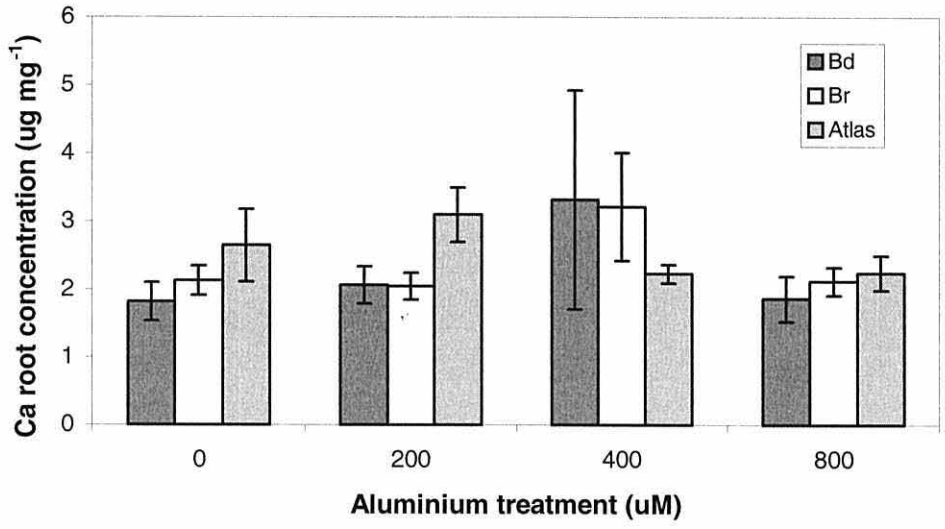

Figure 4.19: Calcium root tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5

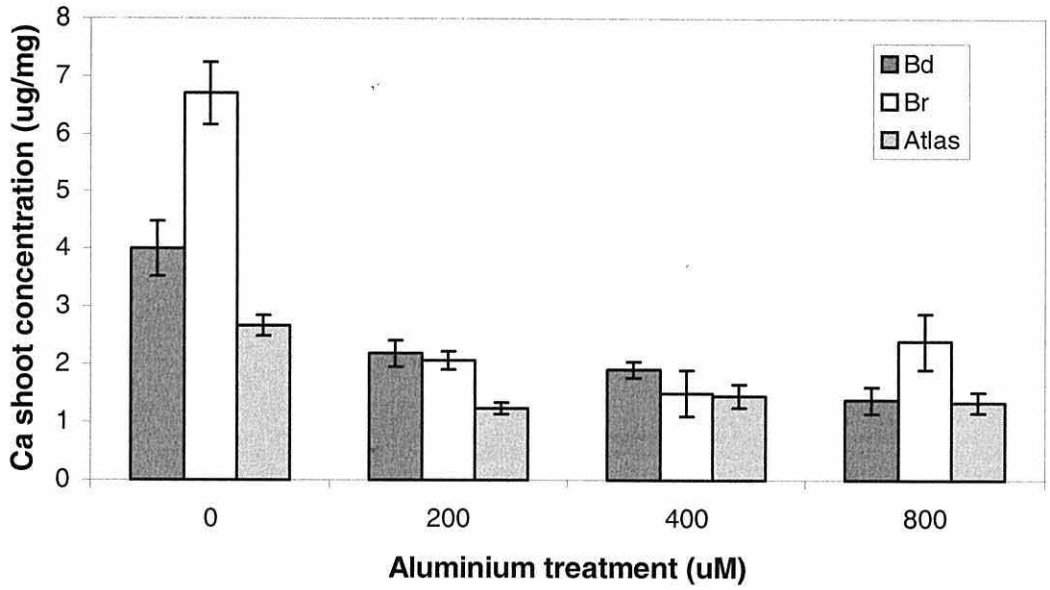

Figure 4.20: Calcium shoot tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n=5

Magnesium root concentration increased with 200 μM aluminium treatment in *Brachiaria decumbens* ($p=0.019$), then decreased back to control levels after treatment with 400 and 800 μM aluminium (Figure 4.21). Magnesium root concentrations were unaffected by 200 and 400 μM aluminium treatment in *Brachiaria ruziziensis*, but were decreased by treatment with 800 μM aluminium ($p=0.045$) (Figure 4.21). *Triticum aestivum* magnesium root concentration was much lower than in either *Brachiaria* species ($p<0.001$), and decreased gradually with increasing aluminium treatment.

Magnesium shoot concentrations were approximately halved in *Brachiaria ruziziensis* ($p<0.001$) and reduced by a third in *Brachiaria decumbens* ($p=0.037$)

after treatment with aluminium (Figure 4.22). Further increases in aluminium treatment did not decrease magnesium shoot concentration further in the two *Brachiaria* species. *Triticum aestivum* magnesium levels were approximately half the level found in either *Brachiaria* species under control conditions ($p=0.045$). Treatment with aluminium decreased magnesium shoot content in *Triticum aestivum* by approximately a third ($p<0.001$) (Figure 4.22).

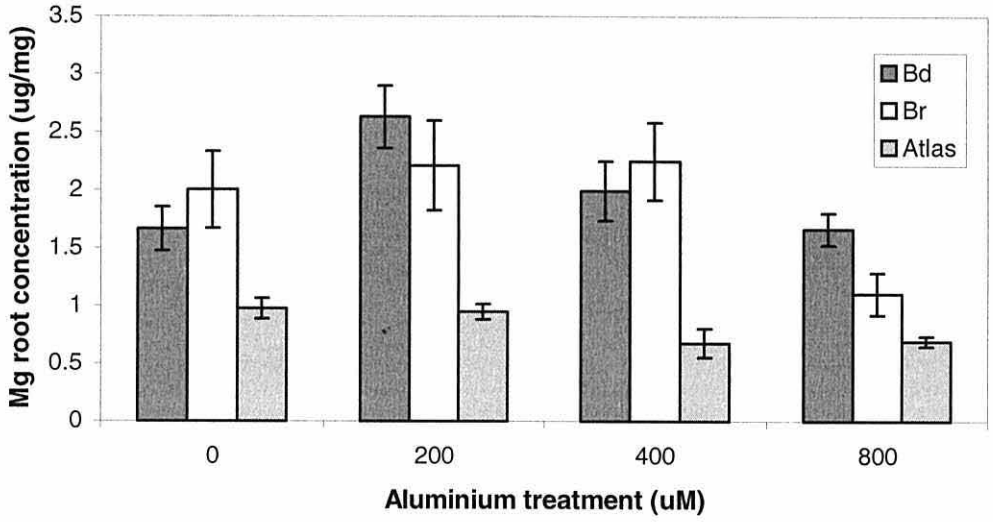

Figure 4.21: Magnesium root tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5

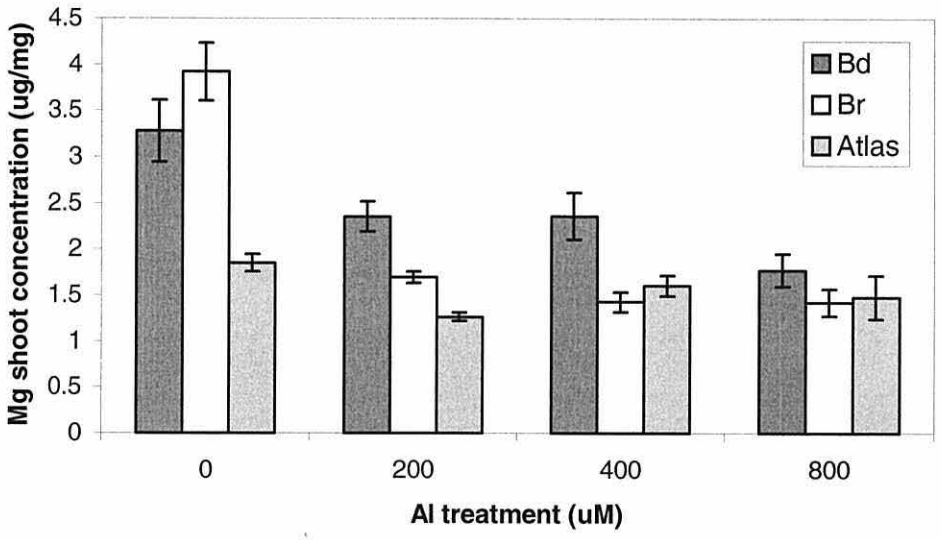

Figure 4.22: Magnesium shoot tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800μM aluminium. ± S.E. n = 5

Potassium root concentrations were approximately six times higher in *Triticum aestivum* than roots of the two *Brachiaria* species ($p<0.001$) (Figure 4.23). There was also a big difference in species response to aluminium, with potassium root concentration decreasing in *Triticum aestivum* ($p=0.018$), increasing in *Brachiaria ruziziensis* ($p=0.093$) and being unaffected in *Brachiaria decumbens* in response to aluminium treatment (Figure 4.23).

Potassium shoot concentrations were again much higher in *Triticum aestivum* than both *Brachiaria* species under control conditions ($p<0.001$), however the potassium concentration decreased in response to aluminium treatment to similar levels found in the *Brachiaria* species (Figure 4.24). The potassium shoot data showed a reverse trend to the root data in the two *Brachiaria* species. Potassium shoot concentration increased in response to aluminium in *Brachiaria decumbens* ($p=0.2$), and remained unaffected in *Brachiaria ruziziensis.*

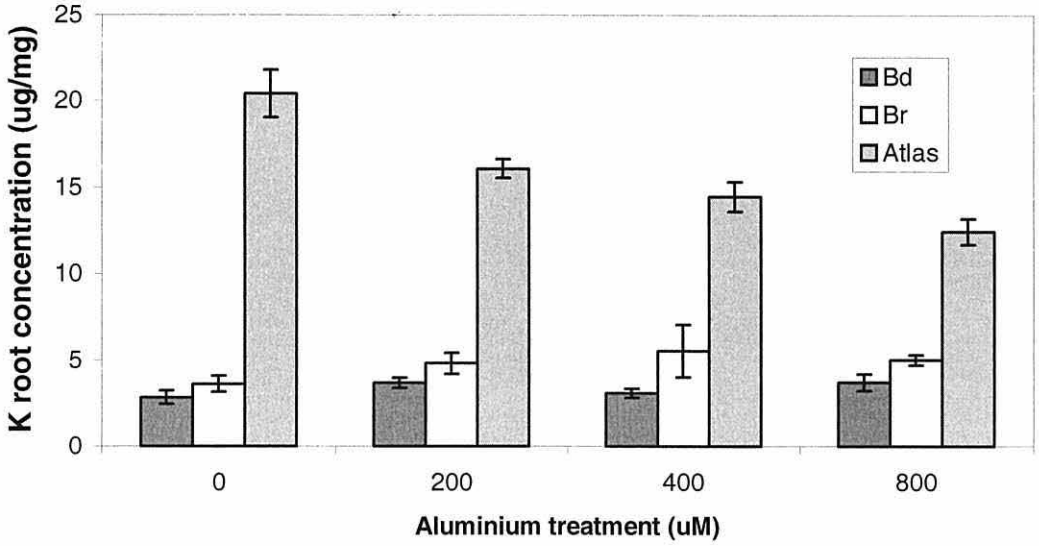

Figure 4.23: Potassium root tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5

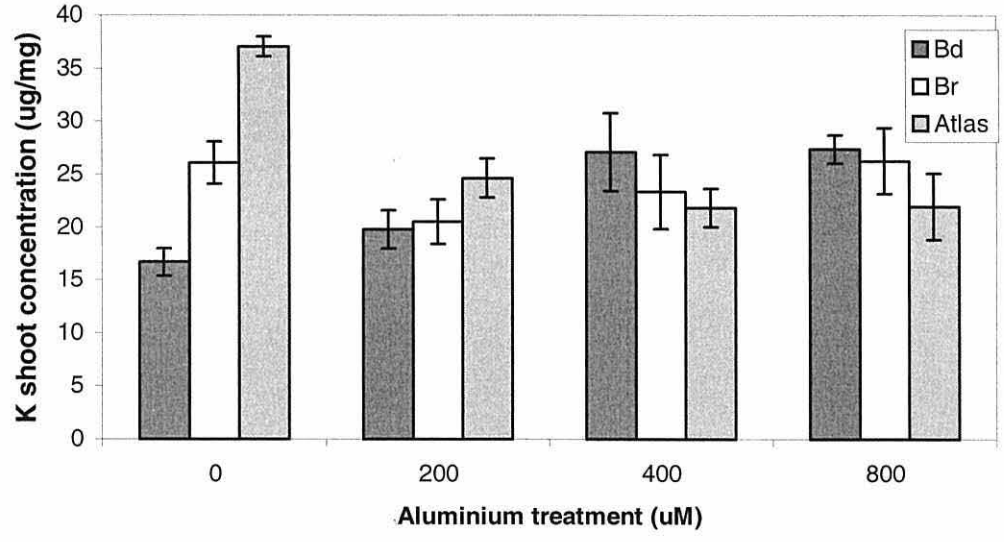

Figure 4.24: Potassium shoot tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800μM aluminium. ± S.E. n = 5

Sodium root concentration followed a similar pattern to potassium, but with much lower levels in *Triticum aestivum* than either *Brachiaria* species (p=0.001) (Figure 4.25). Sodium root concentration increased with increasing aluminium treatment in *Brachiaria decumbens* (p=0.015), decreased with increasing aluminium treatment in *Triticum aestivum* (p=0.009), and was unaffected in *Brachiaria ruziziensis* except after 800 μM aluminium treatment, which caused a decrease in the sodium root concentration (Figure 4.25).

Sodium shoot concentrations were similar in all three species under control conditions (Figure 4.26). Treatment with 200 μM aluminium increased shoot sodium concentration in both *Brachiaria* species, a further increase in aluminium treatment (400 μM) had no further effect on *Brachiaria decumbens* but decreased sodium shoot concentration in *Brachiaria ruziziensis* to levels found in *Triticum aestivum.* 800 μM aluminium treatment caused a decrease in sodium shoot concentration in *Brachiaria decumbens* down to levels found in both *Brachiaria ruziziensis* and *Triticum aestivum* (Figure 4.26). Aluminium caused a decrease in sodium shoot content in *Triticum aestivum.*

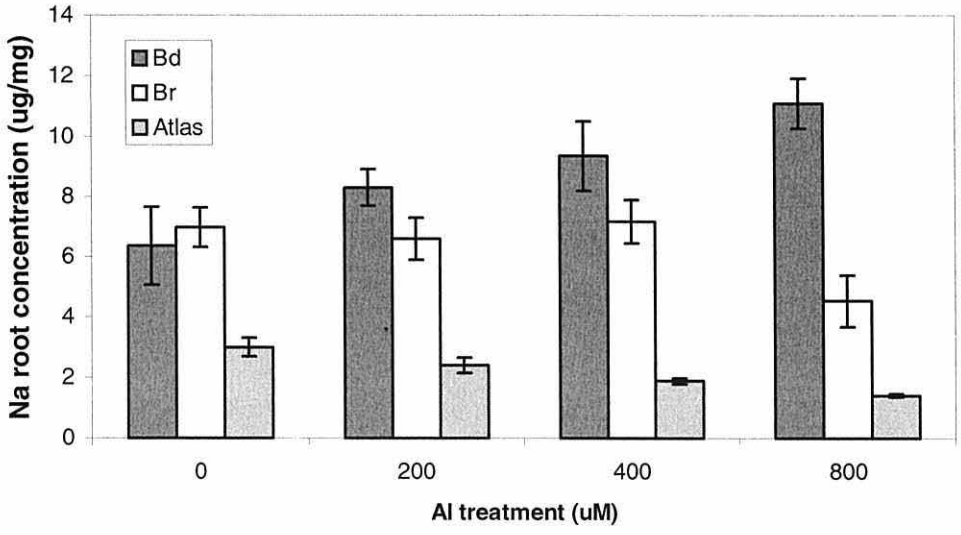

Figure 4.25: Sodium root tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5

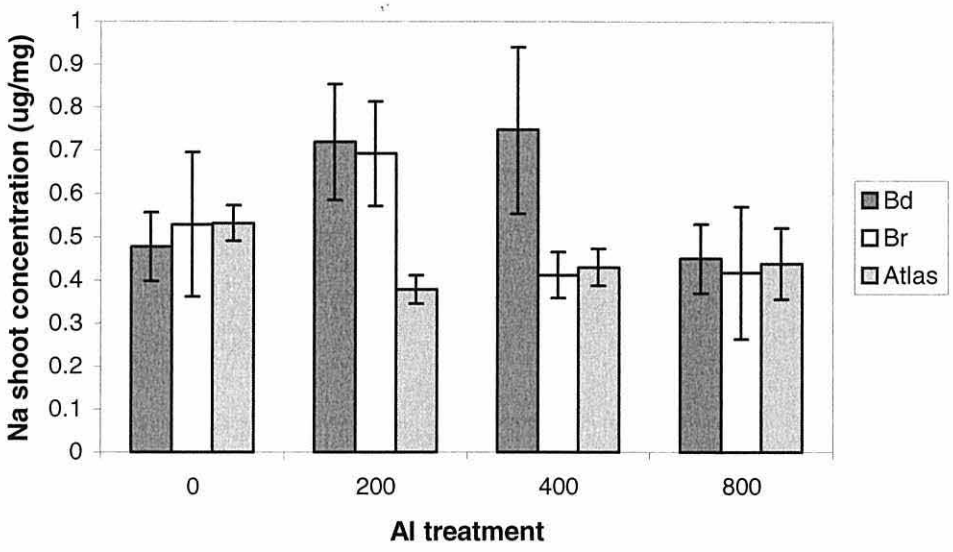

Figure 4.26: Sodium shoot tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n=5

Iron root concentration was higher in *Triticum aestivum* than both *Brachiaria* species under control conditions (p=0.002) (Figure 4.27). Treatment with aluminium caused a decrease in root iron concentration in both *Brachiaria decumbens* (p=0.106) and *Triticum aestivum* (p<0.001). There was also a decrease in iron root concentration in *Brachiaria ruziziensis*, however the trend was not so steep and not statistically significant (p=0.199).

Shoot iron content was twice as high in *Triticum aestivum* than either *Brachiaria* species under control conditions (Figure 4.28). Aluminium treatment halved the iron shoot concentration in *Triticum aestivum* (p<0.001), caused a small decrease in *Brachiaria ruziziensis* (p=0.011), but had no effect on iron shoot concentration in *Brachiaria decumbens*. Iron shoot concentrations were significantly higer in *Brachiaria decumbens* than *Brachiaria ruziziensis* under aluminium treatment (p=0.002).

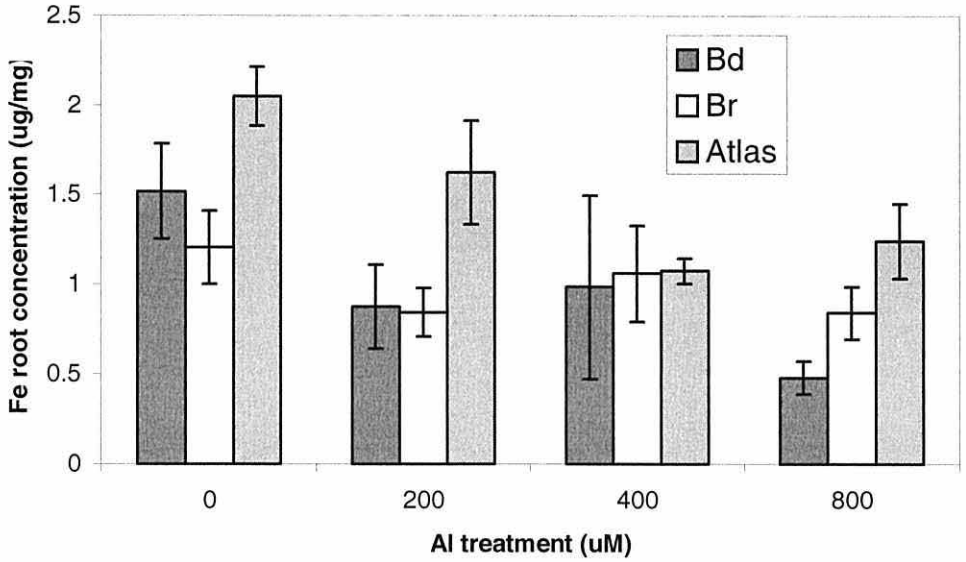

Figure 4.27: Iron root tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5

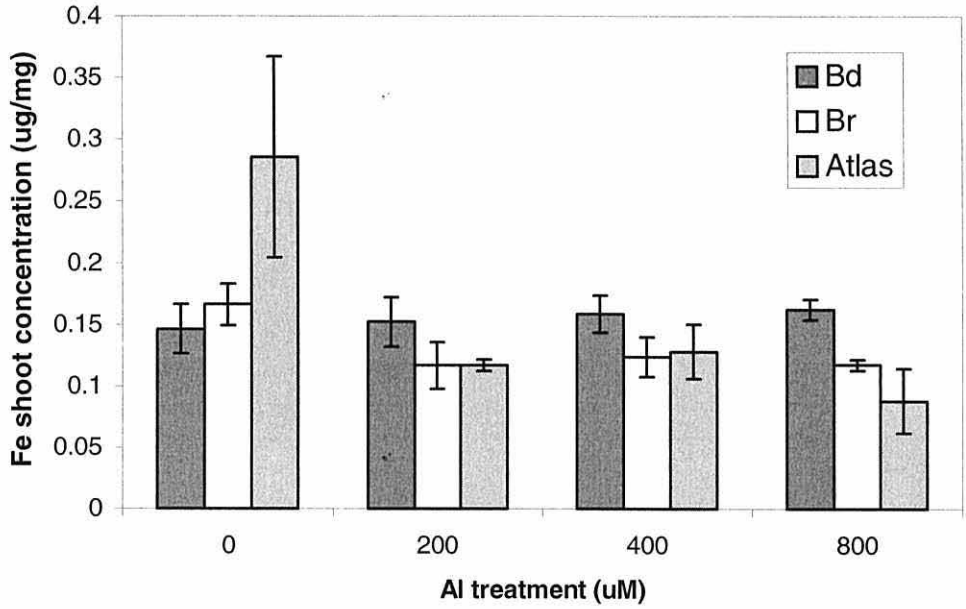

Figure 4.28: Iron shoot tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum var atlas* (Atlas) after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5.

There was a decrease in copper root concentration in response to increasing aluminium treatment in both *Brachiaria* species (Bd p=0.182, Br p=0.003) (Figure 4.29). There was no difference in copper root concentration between the two *Brachiaria* species under any treatment.

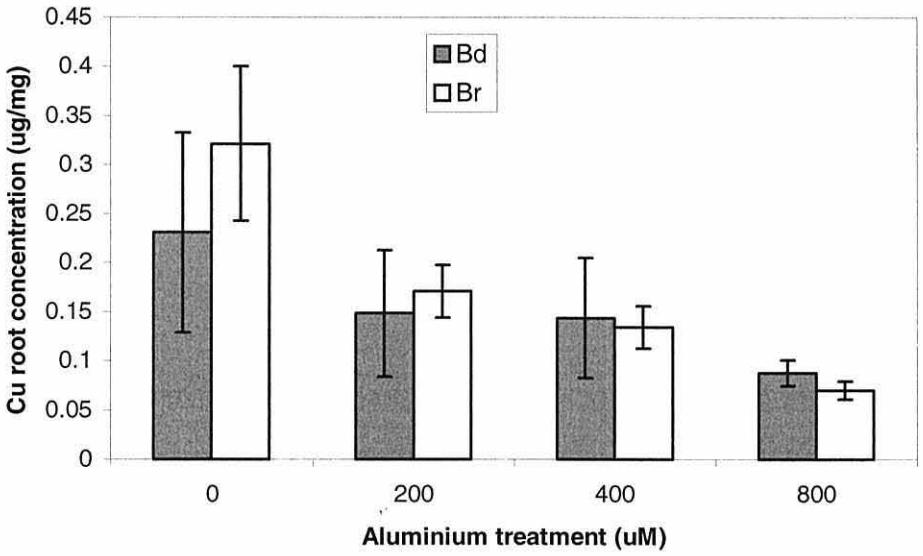

Figure 4.29: Copper root tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br), after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n=5

Aluminium treatment more than halved zinc root concentration in *Brachiaria ruziziensis* (p=0.014) (Figure 4.30). 200 μM aluminium treatment had no effect on zinc root content in *Brachiaria decumbens*, however treatment with 400 and 800μM decreased zinc root concentration down to levels found in *Brachiaria ruziziensis* (p=0.089) (Figure 4.30).

Zinc shoot levels were twice as high in *Brachiaria ruziziensis* than *Brachiaria decumbens* under control conditions ($p=0.036$) (Figure 4.31). Aluminium had no effect on *Brachiaria decumbens* shoot zinc concentration, but halved shoot zinc levels in *Brachiaria ruziziensis* ($p<0.001$), decreasing them down to *Brachiaria decumbens* levels (Figure 4.31).

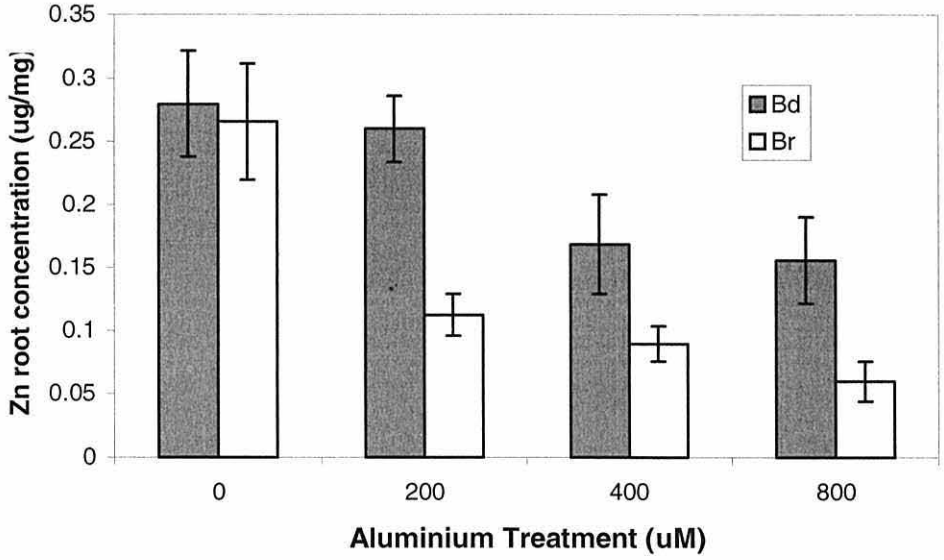

Figure 4.30: Zinc root tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br), after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n=5

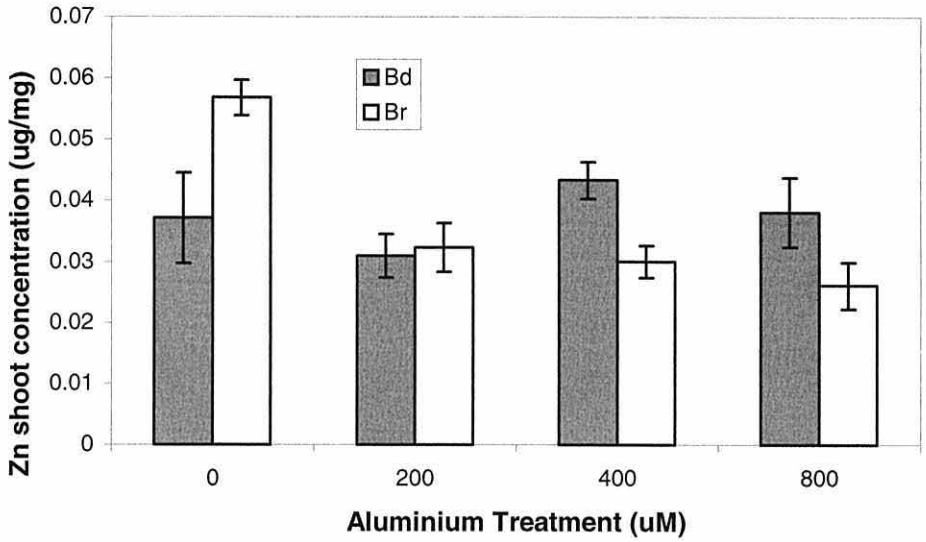

Figure 4.31: Zinc shoot tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br), after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5

Manganese root concentration was higher in *Brachiaria decumbens* than *Brachiaria ruziziensis* (p=0.006) (Figure 4.32). Aluminium halved the manganese root concentration in *Brachiaria ruziziensis* (0.024), and caused a gradual decrease in manganese root content of *Brachiaria decumbens* (p=0.031).

Aluminium treatment halved the manganese shoot concentration in *Brachiaria ruziziensis* (p<0.001) (Figure 4.33), and caused a smaller decrease in manganese shoot concentration in *Brachiaria decumbens* (p=0.034).

Phosphate concentrations were unaffected by aluminium treatment in both *Brachiaria* species, but decreased in *Triticum aestivum* at higher aluminium treatments (200, 400 and 800 μM aluminium) (Figure 4.34).

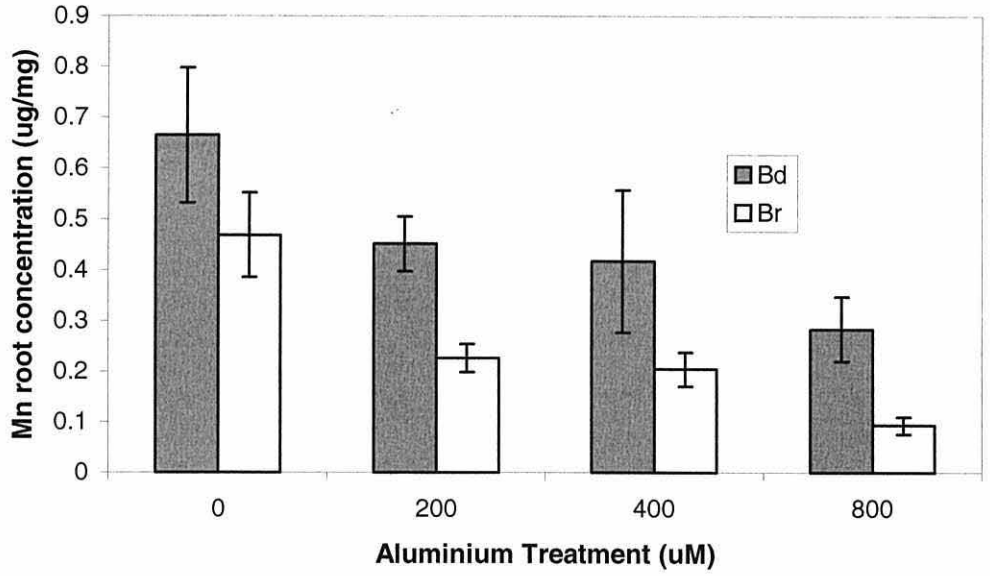

Figure 4.32: Manganese root tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br), after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5

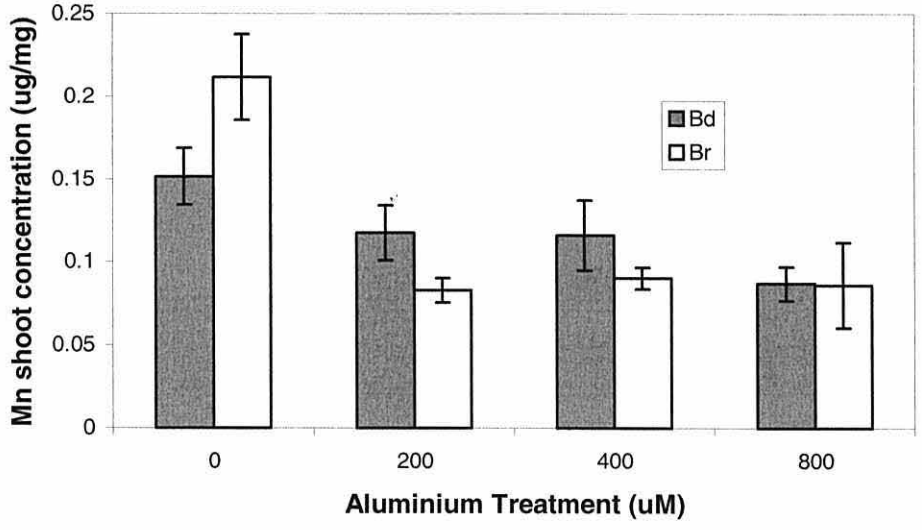

Figure 4.33: Manganese shoot tissue concentration (μg mg^{-1} dry weight) in *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br), after treatment with 0, 200, 400 and 800 μM aluminium. ± S.E. n = 5

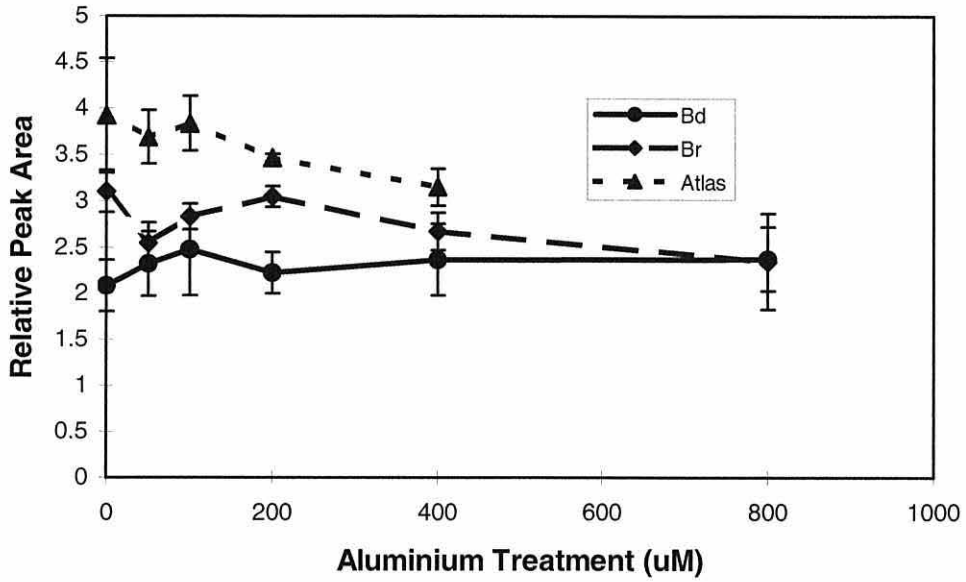

Figure 4.34: Relative phosphate root tissue content in *Brachiaria decumbens* (Bd), *Brachiaria ruziziensis* (Br) and *Triticum aestivum cv atlas* (Atlas), after treatment with 0, 50, 100, 200, 400 and 800 μM aluminium. ± S.E. n = 3.

4.4 Discussion

4.4.1 Experiment One: Effect of nutrition on aluminium tolerance

4.4.1.1 Root growth

The root growth data supports previous studies, which also found that the tolerance of *Brachiaria ruziziensis* to aluminium is dependent on the nutrient level (CIAT, 1998). *Brachiaria ruziziensis* was tolerant to 100 μM Al at high nutrient levels but had reduced root growth with aluminium treatment compared to the control treatment at low nutrient levels. However some other experiments have shown that *Brachiaria ruziziensis* root growth is unaffected by 0.5 mM Al (Osaki *et al.,* 1997), five times higher than the levels used in this study. This discrepancy could possibly be due to the relatively high nutrient levels used in the study by Osaki. The higher the concentration of the nutrient solution, the lower the aluminium levels actually available to interact with plant roots. Solublised aluminium in the nutrient solution

reacts with phosphate and sulphate to form aluminium phosphate and aluminium sulphate, which precipitate out and become unavailable for uptake by the plant.

In nutrient solutions with high calcium and magnesium concentrations there may be competition for apoplastic cation exchange sites between aluminium, calcium and other positive cations such as magnesium. This may decrease aluminium toxicity and the rate of aluminium transport into the symplast. Aluminium solution chemistry is complex and particularly sensitive to pH and availability of hydroxide and other reactive ligands which can bind Al and take it out of solution and unavailable for plant uptake. At pH values above 4.5, aluminium tends to more readily bind to ligands and become less available to plants. This is an even greater problem if the solution contains high concentrations of hydroxide ions either from anions of mineral salts or from adjustment of pH. Hydroxide ions very readily bind aluminium forming a number of aluminium hydroxides, which are thought to be far less toxic than trivalent or polynuclear aluminium. Therefore solutions with high nutrient levels and pH above 4.5 may have less aluminium available to the plant than the amount of aluminium contained in the solution.

There is further evidence to suggest that nutrient levels may have a direct alleviating effect on *Brachiaria ruziziensis* in addition to the effect on aluminium chemistry. Further data showed that 0.2 mM Al had a clear detrimental effect ($p<0.001$) on *Brachiaria ruziziensis* root growth (Chapter Three). Comparing this to the experiment by Osaki *et al.* (1997), where Geochem data showed that the available aluminium concentrations were much higher in the 0.5 μM aluminium treatment (266 μM in the 0.5 M treatment according to Geochem) where it had no effect on the root growth of *B.ruziziensis,* than the 200 μM aluminium treatment (114 μM in the 200 μM treatment according to Geochem). The 0.2 mM Al treatment was at a much lower nutrient level than that of the 0.5 mM treatment. This, added to the trends seen in this experiment and previous pilot studies, provides strong evidence for the presence of a nutrient ameliorating effect on aluminium toxicity. This could be due to a direct ameliorating effect of nutrient(s) such as magnesium and calcium competing with aluminium for apoplastic cation exchange sites and for transport

across the membrane. Nutrient levels could also be influential due to a single nutrient becoming limiting in the low nutrient solutions, increasing the general stress on the plant and so making it less resilient to further stresses such as aluminium stress.

Field studies have found that *Brachiaria ruziziensis* does not grow well when grown in low pH - high Al soils compared to *Brachiaria decumbens*, and eventually dies or is out-competed (CIAT, 1998). This indicates that despite being resistant to relatively high Al concentrations (up to 0.5 mM) under certain laboratory conditions, in natural environments with much lower but continual aluminium exposure, low nutrient levels and competition from better adapted species, *Brachiaria ruziziensis* is unable to survive long term. However this may not be due to a lower tolerance to aluminium than *Brachiaria decumbens*, but could be due to higher nutrient demands, a less efficient nutrient uptake mechanism, lower nutrient uptake per biomass and energy expenditure, lower tolerance to protons and low pH or the culmination of various additive stresses which tip the ecological balance against *Brachiaria ruziziensis*.

4.4.1.2 Root Biomass

The root dry weight data does not mirror the root growth data. Aluminium caused no corresponding decrease in *Brachiaria ruziziensis* root biomass to accompany the decreased root growth at low nutrient levels. *Brachiaria ruziziensis* showed an increase in biomass between the low and high nutrient treatments as opposed to much lower root growth at higher nutrient levels. This could be due to either an increase in branching or a thickening of roots at the higher nutrient levels. *B. decumbens* root biomass is unaffected by either increased nutrient treatment or aluminium treatment, however *Brachiaria decumbens* root growth was much lower under high nutrient than low nutrient conditions. This could again indicate greater branching of side roots or thickening of the roots in the high nutrient conditions.

The difference in response of biomass to nutrient treatment between the two species could well indicate a difference in nutrient utilisation and efficiency between the two species, which may be vital in the low nutrient conditions of low pH soils. *Brachiaria decumbens* does not increase its biomass at higher nutrient levels, suggesting that at low nutrient levels it may already be near its optimum nutrient level, which could indicate a slower growing species better adapted to the low nutrient conditions of a low pH soil. *Brachiaria ruziziensis* increases its root biomass significantly with increased nutrient supply indicating that it is not at optimum nutrient conditions in the low nutrient treatment.

4.4.1.4 Root:Shoot ratio

The root:shoot ratios are clearly different for the two species, *Brachiaria ruziziensis* invests a higher proportion of biomass in roots than *Brachiaria decumbens* under all treatments, indicating that nutrients are more limiting in *Brachiaria ruziziensis* than *Brachiaria decumbens*. This could be due to a more efficient uptake mechanism, or more efficient utilization and lower nutrient demand *in Brachiaria decumbens* than *Brachiaria ruziziensis*. Evidence from the WinRhizo data (Table 2.2) show *B. ruziziensis* to have a smaller fine root diameter than *B. decumbens.* This would give a higher surface area per biomass invested, which in contradiction of the root;shoot ratio data, potentially makes *Brachiaria ruziziensis* the more efficient at nutrient uptake. However, *B. decumbens* has a higher number of root tips per cm of root than *B. ruziziensis* and more branching. The root tips and root hairs are some of the most important root areas for uptake of certain nutrients (Marschner, 1995). The density of transporter proteins in the membranes of root tip cells may also differ between species which would also affect the nutrient uptake capacities of the two species.

The changes in root:shoot ratio with nutrient treatment are as expected. Low nutrient conditions increase the biomass invested in the root system relative to the investment of the shoot system for both species. Interestingly there is also a significant effect of aluminium on the root:shoot ratio of *Brachiaria decumbens* at high nutrient levels ($p=0.021$). Treatment with aluminium increases the investment of biomass in the root system relative to the shoots, possibly indicating a decrease in available

nutrients or ability of the plant to take up available nutrients due to root damage, or compensating for increased root necrosis caused by aluminium toxicity.

4.4.1.5 Aluminium tissue content

The similar aluminium root tip concentrations, combined with the fact that *Brachiaria ruziziensis* exhibited root growth inhibition in response to 100 μM aluminium treatment but *Brachiaria decumbens* did not, shows that aluminium tissue content cannot be used as an indicator of aluminium toxicity. *Brachiaria decumbens* is either able to better exclude aluminium from entering cells, has better internal detoxification or is better able to remediate damage caused by aluminium.

The root aluminium content data includes both symplastic and apoplastic aluminium, most of which will be bound to the root surface and cell walls of the epidermis and outer cortex. The data does not show whether symplastic aluminium is higher in *Brachiaria ruziziensis* than *Brachiaria decumbens*, and so does not show whether the differential aluminium tolerance is due to better exclusion from the cytosol in *Brachiaria decumbens*. However the similar aluminium levels in the shoots of both species may suggest that similar levels of aluminium are entering the symplasm of both species. Internal detoxification would most likely be by binding to organic acids and phosphate, and sequestration in the vacuole. Data for internal organic acid concentrations (Chapter Five) shows no difference between the two *Brachiaria* species, which suggests similar capacity for internal aluminium detoxification between the two species.

There was also no difference in aluminium tissue concentrations of plants grown in high and low nutrient conditions. The calcium and magnesium concentrations therefore had little effect on aluminium binding and uptake by roots of either *Brachiaria* species, although high nutrient levels did ameliorate aluminium toxicity in *Brachiaria ruziziensis.* If the ameliorating effect of magnesium and calcium concentrations on aluminium toxicity were through competition for apoplastic binding sites and competition for transport sites, then aluminium tissue

concentrations would be expected to be lower under high nutrient conditions. This was not the case.

The shoot aluminium concentrations were much lower than root aluminium levels. Aluminium is therefore not readily transported to the above ground biomass unlike in the aluminium accumulator *Melastoma malabathricum*, which sequesters relatively high levels of aluminium in leaves (Watanabe *et al.*, 1998). This is important in restricting movement of aluminium into the human food chain through the use of *Brachiaria decumbens* as cattle fodder and grazing pasture.

4.4.1.6. Root tip cation concentration

The calcium root concentration shows little response to aluminium treatment in either species, however shoot concentration was decreased in *Brachiaria ruziziensis*. This implies that aluminium is not affecting the calcium uptake and distribution in *Brachiaria decumbens* at the treatment levels used. However the decrease in calcium shoot concentration in *Brachiaria ruziziensis* may show a decrease in calcium uptake in the low nutrient medium probably caused by increased competition for transport proteins and an effort to maintain root calcium levels. The decrease in calcium shoot concentration with increasing nutrient treatment may be due to the greater shoot growth seen in the high nutrient treatment.

The magnesium tissue concentration is far more responsive to aluminium treatment than calcium concentration in both *Brachiaria* species, with root concentration increasing in response to aluminium in the low nutrient solution but decreasing in the high nutrient solution, while decreasing in *Brachiaria ruziziensis* shoots at low nutrient treatment.

Both *Brachiaria* species have similar levels of magnesium and calcium in shoot tissue, but magnesium root concentrations are at least twice as high as calcium levels even though the calcium concentration of the nutrient solution is substantially higher than the magnesium concentration. The higher magnesium shoot concentrations in

Brachiaria ruziziensis at high nutrient treatment while no change being seen in *Brachiaria decumbens*, may indicate a higher magnesium demand in *Brachiaria ruziziensis.*

The lower iron levels in *Brachiaria ruziziensis* than *Brachiaria decumbens* under low nutrient conditions indicates a more efficient iron uptake system in *Brachiaria decumbens.* This may be important in soils low in phyto-available iron. In a similar pattern to magnesium, aluminium caused an increase in manganese concentration at low nutrient treatment and a decrease at high nutrient treatment in *Brachiaria ruziziensis.*

4.4.2 Experiment Two: Effect of aluminium dose on tissue nutrient concentrations

4.4.2.1 Aluminium tissue concentration

The root tip aluminium concentration again represents both symplastic and apoplastic aluminium. The gradual increase in aluminium root concentration with increasing aluminium treatment may be due to both increased internal sequestration and excluded apoplastic aluminium. The aluminium shoot concentrations suggest that the two *Brachiaria* species are far better at excluding and sequestering aluminium in the roots than *Triticum aestivum.* The similar root and shoot aluminium concentrations between the two *Brachiaria* species indicates that they have similar exclusion and sequestration capacities.

Plants, such as *Melastoma malabathricum,* able to accumulate 1000 mg kg^{-1} or more aluminium, are termed aluminium accumulators (Chenery, 1948). Both *Brachiaria* species fall far short of this level with accumulation of 6 μg mg^{-1}.

4.4.2.2 Cation tissue concentration

The root tissue cation data shows aluminium clearly has an effect on nutrient uptake. The nutrients can generally be grouped into four sets according to their role and the response aluminium has on their uptake in the three species. Group one contains the major divalent nutrients calcium and magnesium, where shoot concentration is significantly decreased in response to aluminium in all three species while root levels are fairly well maintained, and with little species differences despite their widely differing aluminium tolerances. The second group comprises the monovalent ions potassium and sodium, uptake of which is increased to both root and shoots in both *Brachiaria* species but decreased in the much more sensitive *Triticum aestivum.* Group three comprises iron, where the high root concentrations are decreased in all three species while the much lower shoot concentrations are maintained at control levels in the two *Brachiaria* species but not in *Triticum aestivum.* Group four contains the micronutrients zinc, copper and manganese, again uptake of which is generally decreased with aluminium treatment, but uptake is significantly better maintained in the most tolerant species *B. decumbens* in comparison to *B. ruziziensis.*

The nutrients can also be placed into three different groups based on their differing root:shoot nutrient allocations. Group one again comprises calcium and magnesium both with similar levels in root and shoot concentrations. Group two contains potassium and zinc, with much higher concentrations in shoots than roots. Group three contains iron and manganese, with much higher concentrations in roots than shoots.

Most previous studies have found that calcium and magnesium shoot and/or root concentrations are decreased with aluminium treatment in sensitive cultivars and species, but unaffected or decreased to a lesser extent in tolerant cultivars and species (Jan, 1991; Ohki, 1983; Asp *et al.*, 1991; Watanabe *et al.*, 1997; MacDiarmid and Gardner, 1996; Bernal and Clark, 1997). It is thought that aluminium reduces the apoplastic calcium and magnesium pool by taking over cation exchange sites (Kinraide, 1998) or by blocking calcium channels (Jones *et al.,* 1998a). Here a similar pattern is seen in all three experimental species. Aluminium causes a

decrease in shoot concentrations although root concentrations are fairly well maintained. In response to decreased uptake of calcium and magnesium, both *Brachiaria* species appear to be decreasing shoot calcium and magnesium concentrations to maintain root concentrations. This could either involve decreased transport to the shoots or active transport from shoots to roots. Calcium and magnesium, as major nutrients, are important in maintaining cell turgor, enabling cell expansion and hence root elongation. This therefore represents a possible mechanism for ameliorating nutrient stress caused by aluminium.

A similar explanation may be put forward for potassium. Potassium is at a much higher concentration than either calcium or magnesium in both shoots and roots and is therefore far more significant in maintaining cell turgor. With potassium however, root concentration is not maintained at the expense of shoot concentration. Instead aluminium causes a considerable increase in potassium uptake in *Brachiaria decumbens*, whereas aluminium causes a decrease in potassium uptake in the sensitive *Triticum aestivum.* Increased potassium uptake would therefore appear to be a clear mechanism for aluminium tolerance in *Brachiaria decumbens.* In *Brachiaria ruziziensis,* the slightly less aluminium tolerant of the two *Brachiaria* species, the increase in root potassium concentration may be more at the expense of shoot concentrations rather than increased uptake. This may play a part in the more aluminium sensitive nature of *Brachiaria ruziziensis* than *Brachiaria decumbens.*

Aluminium has been shown to cause a decrease in iron uptake in a number of species (Chang *et al.*, 1998; Lasseigne *et al.*, 1997; Pintro *et al.*, 1998; Brown and Jones, 1977; Bernal and Clark, 1997). Here a similar pattern is seen in all three experimental species, with aluminium causing a decrease in iron root concentration. This may be due to inhibition of both synthesis and exudation of phytosiderophores involved in iron acquisition (Chang *et al*, 1998). Iron root tip concentration was much higher in *Brachiaria decumbens* than either of the other two species. This indicates a superior iron acquisition system, which would give *Brachiaria decumbens* a significant advantage in iron poor soils. *Brachiaria decumbens* was

also better able to maintain iron shoot concentrations, this again indicates better aluminium tolerance.

A much clear pattern is seen in response of micronutrients to increasing aluminium treatment. Root copper and root and shoot zinc and manganese concentrations correspond well with effect of aluminium on *Brachiaria* root growth, decreasing with increasing aluminium treatment This is broadly in line with the other studies in which the general consensus shows a decrease in various micronutrients (Lasseigne *et al.,* 1997; Bernal and Clark 1997; Simon *et al.*, 1994). However some previous studies have found an increase particularly in root copper concentration in response to aluminium treatment (Pintro *et al.*, 1998; Simon *et al.,* 1994). The decreased concentration of micronutrients in both root and shoot tissue is greater in the less aluminium tolerant *Brachiaria ruziziensis* than *Brachiaria decumbens.*

Root phosphate concentrations were unaffected by aluminium treatment in both *Brachiaria* species and *Triticum aestivum*, decreasing slightly in *Brachiaria* ruziziensis and *Triticum aestivum* under exceptionally high aluminium treatments (400 and 800 μM). This broadly follows reports in the literature where phosphate concentration is often found to increase or remain steady in response to aluminium treatment (Pintro *et al.,* 1998; Simon *et al.*, 1994; Mukherjee and Asanuma, 1998; Lasseigne *et al.*, 1997). Increases in symplastic phosphate and increased exudation have been shown to occur in a number of species (Mukherjee and Asanuma, 1998). This pattern of phosphate mobilisation may play an important role in excluding and sequestering aluminium as part of a plant tolerance strategy. The root tip phosphate concentration is significantly higher in *Triticum aestivum* than either *Brachiaria* species. In *Triticum aestivum* phosphate exudation is thought to occur as part of the aluminium tolerance mechanism (Pellet *et al.*, 1996). The higher phosphate concentrations in *Triticum aestivum* may indicate that phosphate accumulation and exudation may be more important to *Triticum aestivum* than it is in *B. decumbens.* However it may also indicate a higher phosphate requirement in the cereal crop than the two *Brachiaria* species.

4.5 Conclusions

The superior adaptation of *Brachiaria decumbens* to nutrient poor acid soil conditions than the closely related *Brachiaria ruziziensis,* is probably due in part to a better nutrient uptake system particularly iron and lower optimum nutrient requirements. It is also probably due in part to better maintenance of root and shoot nutrient concentrations when exposed to aluminium.

The differential *Brachiaria* species aluminium tolerance is not due to difference in aluminium uptake to roots or shoots.

Chapter Five: Aluminium Effect on Organic Acids

5.1 Introduction

5.1.1 Role of organic acids in plants

Low molecular weight organic acids (See Figure 5.1 for chemical structures of common organic acids) are a major metabolic component of plants, concentrations being in the region of 1.5 to 20 mM in leaf cells (Gabriel and Kesselmeier, 1999), and root content typically in the range 10 to 20 mM (Jones, 1998). This is on a par with other metabolic constituents such as sugars and amino acids. They are important photosynthetic intermediates, making them central to cell metabolism (Ivanishchev and Kurganov, 1992; Maheshwari *et al.,* 1992). The concentration and composition of organic acid pools in plants varies, depending on species, photosynthetic mechanism (C3, C4 or CAM), tissue, age, diurnal rhythms, and environmental stresses the plants are exposed to.

Organic acids are involved in a number of metabolic and biochemical processes in plant cells including energy production, carbon reservoirs for C4 and CAM photosynthesis, synthesis of amino acid precursors, and adaptations to external environment and stresses. Organic acids are a very important factor in a plants interaction with the external environment, making up approximately 33% of exudates (Kraffeczyk *et al.,* 1984).

They are also important as metabolic buffers, balancing fluxes in cytosolic cation content. The concentration of cations in the cytoplasm of cells can be 17 times the levels of anions and organic acids in *Quercus ilex* (Gabriel and Kesselmeier, 1999). Organic acids may be involved in buffering excess calcium and other cations taken up by the cell (Chang and Roberts, 1991; Osmond, 1976).

Organic acids may also have a role as internal detoxicants of the by-products of nitrate reduction. Use of nitrate as the nitrogen source has been shown to trigger an increase in organic acid transport and accumulation in roots, where nitrate reduction takes place (Lopez-Bucio *et al.,* 2000). Organic acids are thought to counteract the build up of toxic hydroxide ions, which cannot be efficiently expelled from the cell.

5.1.2 Organic acid synthesis

Organic acids are mainly produced in the mitochondria during the Krebs (Tricarboxylic acid) cycle, although there is some production in the glyoxysomes during the glyoxylate cycle. Malate can also be synthesised in the cytosol from the carboxylation of PEP to oxaloacetate by PEP carboxylase and reduction of oxaloacetate by malate dehydrogenase to malate. The organic acid pools in the mitochondria are relatively small due to transport of organic acid intermediates out of the mitochondria for use in the cytosol or storage in the vacuole.

Large sustained accumulation and exudation of organic acids would quickly exhaust the carbon pool of the roots. An increase in transport of sugars down the phloem often occurs. The sugars are then converted to required organic acids by the various enzymes. In an experiment by Yang *et al.* (2001) it was found that removal of shoots significantly reduced the induction of citrate exudation in response to aluminium treatment. Yang *et al.* (2001) suggested that this is probably due to the shoots role as a carbon and energy source, although the shoots role as a direct source of citrate could not be ruled out.

Tomato plants grown with aerated carbon dioxide (dissolved inorganic carbon) were found to be more aluminium tolerant than tomato plants not aerated with enriched carbon dioxide (Cramer and Titus, 2001). This ameliorating effect was thought to be due to greater synthesis of organic acids in the carbon dioxide elevated plants, due to the increased carbon availability in the roots. Organic acids from carbon dioxide

enriched roots were found to contain much higher concentrations of ^{14}C. This indicates the importance of carbon transport for organic acid synthesis.

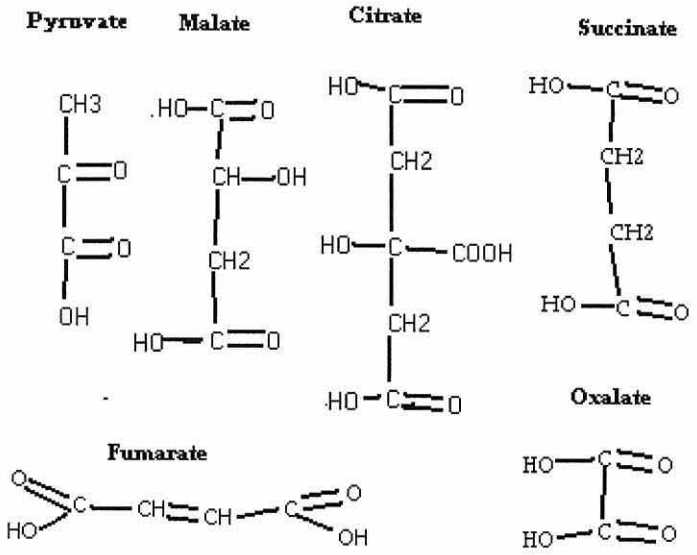

Figure 5.1: Chemical structures for common organic acids found in plants. C = carbon, H = hydrogen, O = oxygen.

Increases in organic acid accumulation and exudation can be caused by increasing the activity or transcription of several enzymes, for example citrate synthase (acetyl CoA + oxaloacetate to citrate) (Mugai *et al.*, 2000), pyruvate kinase (PEP to pyruvate), PEP carboxylase (PEP to oxaloacetate) and isocitrate dehydrogenase (isocitrate to oxoglutarate). Stresses such as P deficiency, aluminium toxicity and iron deficiency have been shown to cause increased transcription of these enzymes (Lopez-Bucio *et al.*, 2000). However de novo synthesis of organic synthesis enzymes does not always correlate with increased exudation (Jones, 1998). Experiments on aluminium toxicity in sensitive and tolerant wheat cultivars found that enzyme concentration was the same in both sensitive and tolerant cultivars, however organic acid exudation was much greater from roots of the tolerant cultivar (Ryan *et al.*, 1995a). The increase in accumulation and exudation is a very rapid response and is therefore probably due not to increased enzyme concentration but

increased enzyme activity and increased transport across the plasma membrane (Ryan *et al.,* 1995a).

5.1.3 Organic acids and nutrient stress

Organic acids have been found to have a role in both increasing uptake of limited nutrients and detoxifying excess nutrients and toxic elements in the soil. Organic acid exudation is induced by a number of nutrient deficiencies including phosphate (Gardner *et al.*, 1983; Lipton *et al.*, 1987), iron (Lopez-Millan *et al.,* 2001; reviewed in Abadia *et al.,* 2002), nitrogen (Franco *et al.,* 1991), zinc (Rengel, 2002) and potassium (Kraffczyk *et al.,* 1984) and general nutrient deficiency (Jones and Darrah, 1995; Rengel, 2002). Coupled to this, an increase in soil acidification by increased activity of membrane bound proton pumps is induced by various nutrient stress conditions (Cohen *et al.,* 1997). General nutrient deficiency has been found to increase organic acid exudation by up to 33 times base levels (Jones and Darrah, 1995)

5.1.3.1 Phosphate deficiency

After nitrogen, phosphorus is typically the next most limiting nutrient deficiency in agricultural soils, particularly in developing countries. Phosphate starvation and deficiency have been shown to cause an increase in organic acid production, accumulation and exudation in a number of species including *Lupinus albus* (lupin) (Gardner *et al.*, 1983), *Medicago sativa* (alfalfa) (Lipton *et al.*, 1987) and *Brassica napus* (rape) (Hoffland *et al.,* 1989). In low pH soils phosphate reacts with aluminium and iron forming insoluble precipitates or is adsorpted to soil particles and so becomes unavailable for plant uptake. At higher pH levels, phosphate in the soil forms calcium and magnesium salts with low availability to plants. Organic acids have a very high affinity for bi and tri-valent cations such as aluminium, iron, magnesium and calcium, they can solublise the phosphate bound to adsorption sites in the soil matrix, thus releasing the phosphate and making it available for uptake by

plants (Gardner *et al.*, 1983; Dinkelaker *et al.*, 1989; Hoffland *et al.*, 1992; Gerke, 1992; Bolan *et al.*, 1997). Plants such as *Lupinus albus* are able to produce proteoid roots in response to phosphate deficiency. These are highly branched with high numbers of root tips, which produce very high concentrations of organic acids and protons to aid phosphate uptake (Gardner *et al.*, 1983). Organic acid exudation can occur all along root length in response to phosphate deficiency, particularly in sections of root in contact with rock phosphate crystals (Gardner *et al.*, 1983). This compares with the situation during aluminium toxicity where exudation is highest around the root tip and apex (Jones and Darrah, 1995, Kochian, 1995), which is thought to be the most important site of aluminium toxicity (Ryan *et al.*, 1993). This implies very precise control of exudation depending on stress and site of stress.

Studies into the phosphate mobilization efficiencies of various organic acids found the relative order of effectiveness to be (Nagarajah *et al*, 1970):-

Good	Moderate	Very poor

citrate > oxalate >> malate, tartarate >> acetate, succinate, lactate

The binding affinity is highly dependent on the number of carboxyl groups (COOH), tri and bi carboxylic acids with three or two carboxyl groups, such as citrate and oxalate, have higher binding affinities than organic acids with only one carboxyl group. The carboxyl group can dissociate losing a hydrogen ion and so gain a negative charge. Tricarboxylic acids therefore have three possible charges, the degree of dissociation depending on concentration, cation concentration and pH. Binding affinity decreases with increasing pH (Parker *et al.*, 1995).

Not all plant species show an organic acid response to phosphate starvation. A good example is shown by *Cassia tora* which is unaffected by phosphate starvation and treatment with La, but organic acid exudation is stimulated by other stimuli such as aluminium treatment (Ma *et al.*, 1997c). This suggests that the stimulation of

organic acid secretion is not a general response to stress but requiring specific triggers, which vary depending on species and conditions. The organic acid response to phosphate deficiency does not appear to occur in graminoid species and has so far only been found in dicots (Haynes and Mokolobate, 2001).

5.1.3.2 Iron deficiency

Iron deficiency is frequently limiting, particularly in drier or more alkaline soils where iron is found predominantly in its less soluble Fe^{3+} form (Hughes *et al.,* 1992). Many plant species highly adapted to low available iron conditions are found to exude high concentrations of various organic acids, this is known as Strategy 1 and occurs in dicots and non graminoid monocots (Romheld, 1987; Jolley *et al.,* 1996; Hughes *et al.,* 1992). Dicots exposed to iron starvation were found to increase organic acid exudation and increase acidity of rhizosphere by increase H^+ pumping. The organic acids have a very high affinity for Fe^{3+} ions. They bind the Fe^{3+} ions, these are then reduced at the cell membrane to Fe^{2+}, which can then be taken up by the plant along the electrochemical gradient (Romheld, 1987; Jolley *et al.,* 1996; Hughes *et al.,* 1992). Interestingly increased organic acid exudation during iron deficiency occurs all along the root length except at the apex, the complete reverse to the pattern seen in response to aluminium toxicity (Welch *et al.,* 1993).

5.1.3.3 Calcium Toxicity

In alkaline/chalky soils, in addition to phosphate and/or other nutrient deficiencies calcium levels can be so high that it becomes phytotoxic. In plants adapted to calcium soils (termed calcicole) this toxicity can be counteracted by either excluding calcium from the symplast in the case of calciphobes. This is often by chelating the calcium with organic acids exudated from the root, particularly oxalate (Cannon *et al.*, 1995). Of all the organic acids oxalate precipitates readily in the presence of calcium, forming Ca-oxalate. This makes oxalate particularly important for phosphate acquisition and calcium toxicity in chalky soils (Cannon *et al.*, 1995; Griffiths *et al.*, 1994). The other calcium tolerance mechanism, employed by calciphiles, involves binding of calcium to oxalate or malate within the symplast.

The Ca-oxalate crystals are then stored in the vacuoles of leaf, root and stem cells (Cannon *et al.,* 1995; Griffiths *et al.,* 1994).

5.1.3.4 Plant/Microbe interaction

Microbial and fungal interactions are extremely important in nutrient acquisition. They are involved in breakdown of complex organic matter to available nutrients, changing insoluble nutrients into forms available for uptake by plants, nitrogen fixation and increased nutrient acquisition by mycorrhiza. Soil micro-biota depend on simple organic compounds including organic acids as an energy source. Microbial growth is therefore higher at the root/soil interface (rhizosphere) than further from the roots. It is thought that exudation of organic acids plays some role in maintaining this gradient of beneficial microbes. Increasing exudation of organic acids and other carbon compounds increases the microbial biota around the roots (Jones, 1998).

5.1.4 Other possible organic acid roles

Organic acids may also be involved in weathering (Lundstrom, 1994). Citrate may play a role in potassium mobilisation (Wallander and Wickman, 1999).

Oxalate retained in hyphal mats may be involved in increasing sulphate availability and calcium oxalate crystals in the soil may act as a reservoir for calcium (Dutton and Evans, 1996). Oxalate may be involved in pathogenesis mechanisms, causing acidification of tissues and removing calcium from walls by forming Ca-oxalate (Dutton and Evans, 1996).

5.1.5 Organic acids and aluminium toxicity

Organic acids have been shown to alleviate aluminium toxicity in a wide variety of plant species including *Triticum aestivum* (Ryan *et al.,* 1993), *Zea mays* (Bartlett and Riego, 1972), *Gossypium hirsutum* (cotton) (Hue *et al.,* 1986), *Lolium perenne* (perennial ryegrass) (Muchovej, *et al.,* 1988), *Fagopyrum esculentum* (buckwheat) (Ma *et al.*, 1997a; Ma *et al.,* 1998; Zheng *et al.*, 1998), *Cassia tora* (Ma *et al.,*

1997c), *Phaseolus vulgaris* (French bean) (Mugai *et al.*, 2000), *Glycine max* (Yang *et al.*, 2000) and *Sorghum bicolour* (sorghum) (Shuman *et al.*, 1991). Aluminium has been shown to increase exudation of organic acids, in *Triticum aestivum* (Delhaize *et al.*, 1993b; Pellet *et al.*, 1996), *Cassia tora* (Ma *et al.*, 1997c), *Arabidopsis thaliana* (Larsen *et al.*, 1998) and *Fagopyrum esculentum* (Ma *et al.*, 1998).

The organic acids are able to chelate aluminium preventing the aluminium from binding to cell walls or plasma membranes. This prevents the aluminium from crossing the plasma membrane into the cytosol, thereby making it non-toxic to plants. Malate may be able to alleviate aluminium toxicity by preventing the blocking of plasma membrane calcium channels by aluminium (Huang *et al.*, 1996).

Different species have been shown to secrete different dominant organic acids. *Fagopyrum esculentum*, an extremely aluminium tolerant species, uses oxalate as the dominant organic acid, both for internal detoxification (Ma *et al.*, 1998) and exudation (Ma *et al.*, 1997a; Zheng *et al.*, 1998). *Triticum aestivum* has been shown to exude malate (Ryan *et al.*, 1995b). *Phaseolus vulgaris* (Mugai *et al.*, 2000) and *Glycine max* (Yang *et al.*, 2000) secrete citrate in response to aluminium treatment. Aluminium toxicity studies on 10 herbs and grasses found that *Rumex acetosella* and *Viscaria vulgaris* increased exudation of oxalate in response to aluminium. *Galium saxatile* and *Veronica officinalis* increased citrate exudation (Schottelndreier *et al.*, 2001).

The induction of increased organic acid exudation in response to exposure to aluminium is a rapid mechanism and can take less than 30 minute (Ma *et al.*, 1997b, Zheng *et al.*, 1998b). It has also been shown to be specifically induced by the trivalent aluminium form (Al^{3+}) in *Triticum aestivum*, rather than other aluminium species i.e. aluminium hydroxides (such as $Al(OH)_3$) or Al_{13} (Ryan *et al.*, 1995).

The continued induction of increased organic acid exudation depends on continual exposure to toxic aluminium. Removal of *Triticum aestivum* roots from aluminium containing solutions causes the organic acid exudation to decrease to pre-treatment levels (Ryan *et al.,* 1995). Further evidence comes from Yang *et al.* (2001). They showed that aluminium causes an increase in exudation of citrate from root tips of *Glycine max.* Increased secretion occurred after four hours of aluminium treatment. Treatment of only part of the root system using a split chamber set up showed that only the parts of the root treated with aluminium increased citrate exudation, thus showing the need for direct aluminium interaction with the root.

Increase in exudation in response to aluminium treatment is highest at the root apex (Kochian, 1995; Jones and Darrah, 1995). This is completely different to the response pattern triggered by phosphate and iron deficiency (Welch, 1993). The apex is thought to be the site of aluminium toxicity (Ryan *et al.,* 1993), therefore higher exudation at the root apex would be the logical plant response.

Evidence for the role of organic acids in aluminium tolerance is found in a large number of studies such as Larsen *et al.*, (1998), who found that in aluminium resistant mutants of *Arabidopsis thaliana*, the aluminium resistance was located on two chromosomes, numbers 1 and 4. All the mutants were found to accumulate less aluminium in the root tips compared to the sensitive wildtype, indicating the possible involvement of an exclusion mechanism. Further investigation found that mutants with the mutation on chromosome 1 exuded much higher levels of citrate and malate than wildtype, indicating that the increased aluminium and tolerance of these mutants was due to organic acid chelation. Chromosome 4 mutants did not show increased organic acid exudation compared to wildtype, but did show increased alkalisation of the surrounding solution.

Work on *Fagopyrum esculentum* exudation has shown an increase in oxalate secretion with increasing aluminium treatment (Zheng *et al.,* 1998b). Oxalate exudation occurred within as little as 30 minutes of exposure (Ma *et al.*, 1997b).

Treatment with phenylglyoxal reduced oxalate secretion by 50%. Root elongation was not inhibited by 25 μM aluminium or 10 μM phenylglyoxal, but was inhibited by 40% when treated with both, indicating the importance of oxalate in the aluminium tolerance mechanism of *Fagopyrum esculentum* (Zheng *et al.*, 1998b).

Colocasia esculenta (Tara) secretes higher concentrations of oxalate in response to aluminium exposure (Ma and Miyasaka, 1998). The response occurred within 3 days, increasing linearly then reaching a plateau after 5 days. The higher the aluminium treatment, the more oxalate was secreted. This response was found to be aluminium specific and not triggered by phosphate starvation (Ma and Miyasaka, 1998).

Species distribution studies in the field found that species with high organic acid exudation colonized the low pH soil areas (more aluminium toxic), and species with low exudation were confined to areas with more neutral pH (Schottelndreier *et al.*, 2001). This indicates that organic acids may play a very important role in defining the ecological niche of plant species.

Few investigators have looked at the longer term effects on organic acid efflux, however Zheng *et al* (1998a) carried out longer term experiments on a number of sensitive species and showed three separate responses to Aluminium. Sensitive species/cultivars showed low organic acid levels throughout the ten-day period. Moderate species/cultivars showed an initial high concentration gradually decreasing over the period. The tolerant *Fagopyrum esculentum* showed continual high rates of organic acid exudation. There were also changes in the organic acid composition over time. In *Brassica napus*, exudation of malic acid rapidly decreased and citric acid increased from a negligible level. Increasing aluminium concentrations increased the exudation of citrate in *Cassia tora*, with longer exposures increasing the efflux of citrate (Ma *et al.*, 1997c).

5.1.5.1 Mechanisms

Basu *et al.* (1994) has suggested that the increase in malate exudation in response to aluminium treatment was due to an increase in both malate synthesis and increased transport across the membrane. The speed of response of increasing exudation, within 20 minutes of exposure to Al in *Triticum aestivum* (Delhaize *et al.,* 1993b, Ryan *et al.,* 1995), means it is more likely that the increased synthesis is at least initially due to increased activity of the various enzymes rather than an induction of genes and an increase in enzyme concentration. However, work on *Cassia tora* has shown that there was a 4-hour lag in the increased secretion of citric acid in response to aluminium, and that secretion continued for several hours after removal from the aluminium solution (Ma *et al.,* 1997c). This directly contradicts the findings in *Triticum aestivum* and may implicate the involvement of de novo synthesis of enzymes involved in the synthesis of citric acid.

5.1.5.2 Transport across membranes

Increase in root tissue organic acid levels does not always correspond with an increase in exudation. Bulk tissue organic acid measurements will reflect the organic acid concentration in the vacuole, which makes up to 90% of the volume of root cells depending on age and distance from the root apex. Exudation will depend on the organic acid concentration gradient between the cytosol and apoplast, not on the vacuolar concentration. The concentrations of the cytosol remain reasonably constant, rarely exceeding 5 mM, due to the regulatory role some organic acid concentrations have on metabolic activity (Chang and Roberts, 1989; Chang and Roberts, 1991).

The organic acid concentration of the apoplast can be up to 1000 times less than the concentration in the cytosol (cytosol 0.5 to 10 mM, apoplast 0.5 to 10 μM) (Jones, 1998). In addition, a large electrical potential gradient caused by the pumping of protons out of the cytosol into the apoplast, results in a large electrochemical gradient to power the passive movement of organic acids, such as citrate and malate, into the rhizosphere (Jones, 1998).

Two mechanisms for organic exudation have been suggested. The first involves passive diffusion across the lipid bi-layer down the electrochemical gradient. The rate of this passive diffusion will be increased if the membrane is damaged or made more permeable by calcium or zinc deficiency (Cakmak and Marschner, 1988), common in low pH soils.

The second transport mechanism involves the opening of anion channels, which allow the movement of organic anions through the cell membrane. Delhaize *et al.* (1993b), Ryan *et al.* (1995b) and Ma *et al.* (2001) concluded that the increased efflux of malate from wheat in response to aluminium treatment was due to increased transportation across the membrane by specific anion channels. Gradual damage to the membrane by prolonged aluminium exposure may stop the increased activity of the channels thereby decreasing exudation rate (Zheng *et al.,* 1998a*)*. Further evidence for the role of specific anion channel involvement in control of exudation is derived from experiments involving anion channel antagonists (Ma and Hiridate, 2000).

Experiments on aluminium uptake in *Fagopyrum esculentum* found that aluminium levels in the xylem were 15 times higher in plants exposed to aluminium chloride than aluminium oxalate, thus showing that aluminium is preferentially taken up in its ionic form (Ma and Hiradate, 2000). Respiratory inhibitors had no effect on uptake of aluminium, however lanthanum reduced aluminium uptake, indicating that aluminium uptake is a passive non-energetic process (Ma and Hiradate, 2000).

To maintain electrical neutrality a counter ion is involved in the transport of organic acids across membranes. In iron deficiency the counter ion is thought to be H+. However, exudation of hydrogen ions in aluminium toxic soils would decrease rhizosphere pH, thereby making aluminium more mobile and toxic. Work on wheat found there to be no increase in hydrogen ion exudation with increasing malate

exudation in response to aluminium toxicity (Kochian, 1995), therefore protons are not the counter ions. Ryan *et al.* (1995) found potassium to act as the counter ion.

Johnson *et al.* (1996) found that although succinate made up 34% of the organic acid content of the roots examined, there was no detectable succinate in the exudates. This argues against a large role for passive diffusion, and argues for control of organic acid composition of the exudates by selectivity of the anion channels. In opposition to this are theoretical calculations of passive diffusion of malate based on the concentration and electropotential gradients. These calculations derived a figure for exudation broadly in line with baseline exudation from *Triticum aestivum* (Jones, 1998).

5.1.5.3 Internal Aluminium Detoxification

Despite the high toxicity and extreme reactivity of aluminium, it has long been known that some species e.g. *Camellia sinensis* (Matsumoto *et al.*, 1976) and *Hydrangea chinensis* (Ma *et al.*, 1997a) are able to accumulate aluminium to very high concentrations in the leaves. *Camilla sinensis* leaves have been shown to accumulate up to 30,000 mg/kg dry weight in the old leaves, which compares to 50 mg/kg in the more aluminium sensitive *Brassica napus* (Ma *et al.*, 1997b). Localisation of aluminium accumulation in the cells is not yet well determined, although evidence suggests that it may be in the cytoplasm (Cuenca *et al.*, 1990). If so then there must be some very robust mechanism for internal detoxification of the extremely reactive trivalent form of aluminium which would be able to react with a wide variety of cell internal structures despite the pH of cell sap (approximately 7.1 in the cytosol) (Taylor, 1991). Work on *Hydrangea chinensis* has provided evidence for the role of organic acids as internal detoxicants (Ma *et al*, 1997a). Further evidence comes from work on *Fagopyrum esculentum*, which accumulates high concentrations of aluminium in leaves (Ma *et al.*, 1998b). The accumulated aluminium was found to be in the form of aluminium oxalate. NMR studies found the ratio of aluminium:oxalate to be 1:3 (Ma *et al.*, 1997a).

5.1.5.4 Aluminium detoxifying efficiency

Several researchers have looked at the relative aluminium alleviating abilities of the various organic acids. Ginting *et al.* (1998) found that the order of effectiveness was:-

oxalate>citrate=tartrate=malate>malonate>>>lactate

This was calculated by looking at the effect of the various organic acids on root growth rate. It has been suggested that the differing effectiveness of the various organic acids in alleviating aluminium stress is due to their differing stability constants. Al-citrate > Al-oxalate > Al-malate (Zheng, *et al.,* 1998b). In addition to the effect on root elongation, Ginting *et al.* (1998) also investigated the aluminium complexing ability of the organic acids by looking at trivalent aluminium concentrations after addition of the organic acids. Here they found a different order of effectiveness:-

Citrate > oxalate >> tartrate = malate = malonate >>> lactate.

This suggests that the organic acids are protecting the plant from aluminium toxicity in an additional manner than just by chelating/complexing aluminium. Different organic acid concentrations are required to detoxify a particular aluminium concentration, depending on the organic acid used and its chelating ability.

Experimental evidence from researchers such as Parker and Pedler (1998) has shown a discrepancy between the amount of organic acid secreted and the exogenous amounts added to make more sensitive genotypes equally resistant, which can be up to 400% (Parker and Pedler, 1998). However, these calculations rely on the accuracy of estimates of exudation rates, which are not uniform along the root, but are thought to be much higher in the final 2 - 5mm root tip (Ryan *et al.*, 1995, Delhaize *et al.,* 1993b) and around the apical region, which is thought to be the site of aluminium

sensitivity. Models of organic acid soil chemistry suggest that concentrations as high as 50 μM are possible adjacent to the root apex (Jones *et al.*, 1996).

It is now widely thought that although organic acid exudation may be very important in protection against aluminium, the concentrations exuded are insufficient to account for total tolerance to aluminium and therefore other mechanisms must be involved.

5.1.5.6 Humic and Fulvic acids

Experiments on the effect of humic and fulvic acids in alleviation of aluminium toxicity and toxicity caused by the rare earth element lanthanum showed that both were able to alleviate retardation of root growth in *Zea mays* (Diatloff *et al.*, 1998) and *Zea mays* cv DK687 (Harper *et al.*, 1995) caused by low concentrations of aluminium. They concluded that tolerance to aluminium toxicity in soils would be appreciatively higher than suggested by hydroponic studies due to the humic and fulvic acid content of soils. This also suggests that green manures, decomposition of which increases humic and fulvic acids, may help alleviate aluminium toxicity (Harper *et al.*, 1995)

5.1.5.7 Mycorrhizas

Work on *Pinus sylvestris* (Scots pine) seedlings with or without ectomycorrhizal infection have shown that treatment with aluminium causes an increase in oxalate secretion. This increase in oxalate efflux, although seen in both mycorrhizal infected and non-infected root exudates, was much higher in solutions with ectomycorrhizal infected roots (Ahonen-Jonnarth *et al.*, 2000). This indicates mycorrhizas may play an important role in aluminium tolerance and detoxification.

5.1.5.8 Other exudates as aluminium detoxicants

As well as organic acid exudation, phosphate exudation may be part of the mechanism of aluminium tolerance (Pellet *et al.*, 1997). Work on the tolerant

Triticum aestivum cultivar (atlas), a near isoline (ET3) and a sensitive cultivar (scout), showed that although more resistant than scout, ET3 was 3 times less tolerant than atlas (Pellet *et al.*, 1996). Examination of the exudates from the different lines of wheat found that atlas and ET3 both had a large efflux of malate from the root tip compared to scout. In addition atlas was found to exude much higher levels of phosphate than ET3, this could explain the difference in tolerance between ET3 and atlas, phosphate readily binds trivalent aluminium ions (higher binding affinity than citrate) forming insoluble aluminium phosphate, and may also be involved in raising the pH around the root tip found in atlas (Pellet *et al.*, 1997).

5.1.5.9 Importance of organic acids exudation as an aluminium tolerance mechanism

The effectiveness of organic acid secretion to detoxify mobile aluminium in soils may not be as great as many hydroponic studies suggest. Organic acids in the soil can be quickly broken down by microbial action. Experiments using carbon-14 to follow the flow of carbon in conifer soils found that organic acids in the upper organic horizons have a half life of 30 minutes, compared to 8 hours in the lower horizons (Jones *et al.*, 2001) and 2-3 hours in bulk soil (Jones and Darrah, 1994). Aluminium bound organic acid levels are found to be much higher in the upper O (organic) horizon than in the lower horizons (van Hees and Lundstrom, 2001). Mineralisation of organic acids is estimated to be 2 to 3 times faster at the rhizosphere than in the bulk soil (Jones, 1998b). Sorption of organic acids to aluminium and iron hydroxide sites on soil particles may offer some protection from microbial mineralisation (Boudot *et al.*, 1989; Jones *et al.*, 1996b). Aluminium was found to have little effect on the mineralisation of organic acids by microbes except at very high aluminium levels (5 mM).

Despite all the evidence of induction of organic acid exudation and accumulation in response to aluminium treatment, there is still some doubt as to how important organic exudation is as an aluminium tolerance mechanism. Studies looking at the concentrations of exogenous malate required by sensitive *Triticum aestivum* genotypes, exposed to differing aluminum treatments to relieve symptoms of

aluminium toxicity in line with tolerant strains, found that much higher malate concentrations had to be added to the sensitive cultivars than were exuded by the tolerant cultivars (Ryan *et al.*, 1995b; Delhaize *et al.,* 1993b; Pellet *et al.*, 1996; Parker and Pedler, 1998). Estimates of malate exudation from roots of tolerant *Triticum aestivum* cultivars varied from 0.3 μM to 10 μM malate, and in one study it was as high as 25 μM, depending on cultivar and aluminium treatment (Delhaize *et al,* 1993b; Ryan *et al,* 1995b; Pellet *et al,* 1996). Exogenous malate concentrations 4 to 600 (depending on study and conditions) times higher than exudates concentrations from tolerant cultivars are needed to achieve similar levels of tolerance in sensitive cultivars. This indicates that organic acid exudation may only be part of the picture of aluminium tolerance (Parker and Pedlar, 1998). However, due to the sampling methods used in these studies, estimates of malate concentration adjacent to the root cell walls may be low, malate concentrations may decrease with increasing distance from the root/solution interface. Therefore, if sampling some distance from the root then malate measurements will be much lower than those at the root surface.

Another indication of other mechanisms involved arises from the study by Ryan *et al.,* (1995c). Thirty-six wheat cultivars were ranked according to aluminium tolerance, this ranking correlated extremely well with increasing malate exudating ability at low aluminium treatment levels, but not so well at higher aluminium treatment levels (Ryan *et al.,* 1995c). It was suggested that a mechanism other than malate exudation is involved with wheat at higher aluminium concentrations.

5.1.6 Chapter aims

Little research has been conducted into the aluminium tolerance mechanism in *Brachiaria decumbens* or *Brachiaria ruziziensis.* It is not yet known whether the high aluminium tolerance of these species and the differential tolerance between the two species is due to aluminium induced increases in organic acid accumulation and exudation. This chapter will examine the organic acid content of root tissue and exudation to endeavor to answer this question, as well as identify the primary organic

acid involved in the aluminium response and whether organic acid accumulation and exudation is triggered by other stresses such as phosphate deficiency.

5.2 Methods

5.2.1 Experiment One: Spatial distribution of organic acids

Fourteen-day old *Brachiaria decumbens* and *Brachiaria ruziziensis* plants were treated with 0 or 400 μM aluminium chloride, 3 pot replicates per treatment. Plants were harvested for extraction of organic acids from root and shoot tissues after 4 hours of treatment. Four plants sections were chosen to investigate organic acid tissue distribution, 0-1cm from root tip (root tip), 1-2cm from root tip, 5-6cm from root tip (upper root) and leaf blades. Organic acids were extracted by freeze rupturing and analysed by capillary zone electrophoresis.

5.2.2 Experiment Two: Phosphate starvation experiment

Three-week old plants were pre-treated for 24 hours with either nutrient solution minus phosphate, or control nutrient solution containing phosphate. Treatment started with 200 μM aluminium, either with control solution or the equivalent solution without phosphate. Plants were harvested and organic acids extracted after 24 hours of treatment.

5.2.3 Experiment Three: Aluminium dose response experiment

Three-week old plants were treated for 24 hours with 0, 50, 100, 200, 400 or 800 μM aluminium and extract from root tips (0 to 1cm from tip) was analysed for organic acid content. Some plants grown in hydroponic solution formed two differing root morphologies. These were normal, thin root growth with high level of branching, or fast-growing thick vertical roots with less branching. Sample root tips from the two different morphology types treated with 400 μM aluminium were also analysed.

5.2.4 Experiment Four: Organic acid time response experiment

Four-week old plants were exposed to 0 or 200 μM aluminium. Three pots per treatment were harvested after 0, 1h, 2h, 4h, 10h, 24h and 72h and root tips analysed for organic acid content.

5.2.5 Experiment Five: Apoplastic or symplastic aluminium

A small experiment was carried out to investigate whether the citrate released by addition of EDTA was bound to aluminium from within the cell, or apoplastic aluminium bound as the citrate passed through the cell wall and apoplast during the freeze rupturing and sample extraction process. Root tips exposed to 0 or 400 μM aluminium chloride in nutrient solution were subjected to either two thirty second washes in distilled water (usual practise for all experiments) or two 5 minute washes in 800 μM potassium phosphate solution to remove aluminium from apoplastic sites. The root tips were then extracted and analysed for citrate concentration with and without addition of 400 μM EDTA.

5.2.6 Experiment Six: Measurement of exudation

5.2.6.1 Time response experiment

Brachiaria decumbens and *Brachiaria ruziziensis* seeds were germinated and grown on mesh (1 mm) approximately 40 plants per pot. The plants were grown for five weeks using the same control nutrient medium and growth conditions described in Chapter Two. The roots, which completely filled the beakers, were washed in 100μM calcium chloride and transferred to 100 μM calcium chloride as nutrient solution. Calcium chloride was used as treatment solution due to the interference in the analysis of organic acids caused by the full nutrient solution. Organic acids were bound by cations in the full nutrient solution, particularly iron, reducing measurable organic acid peak areas. In addition the relatively high ionic strength of the full nutrient solution further decreased the chance of detecting the low organic acid concentrations found in the exudate. After 24 hours plants were exposed to 0, 50, 100 or 200 μM aluminium chloride in 100 μM calcium chloride solution. 1 ml samples were taken 0, 6, 12, 24, 48 and 72 hours after the start of treatments. 20 μM

molybdate was added to each sample as an internal standard and the organic acid content measured.

5.2.6.2 Pico-sampling

Three-week old *Brachiaria decumbens* plants were placed in petri dishes with 200 μM calcium chloride containing either 0, 200 or 400 μM aluminium. Samples of nutrient solution were taken at the root tip surface using a constricted glass capillary mounted on a 'pressure probe' sampling system (Tomos, 2000). This allowed samples of constant volume of approximately 50 picolitres to be taken. The samples were then placed on a glass slide under oil to prevent loss of sample by evaporation and then analysed using a specially adapted capillary electrophoresis system. Organic acid analysis was carried out using buffer 1 as carrier electrolyte and the electrophoretic conditions described previously. Results were recorded using an analogue pen recorder and relative organic acid concentration measured as peak height.

5.3 Results

5.3.1 Experiment One: Spatial distribution of organic acids

Malate concentrations were similar in the root tip, elongation zone and mature root in *Brachiaria ruziziensis* under control conditions, but higher in the root tip after aluminium treatment ($p=0.121$) (Figure 5.2). In *Brachiaria decumbens* under aluminium treatment, malate concentrations were higher in the root tip than more mature root tissue ($p=0.074$) (Figure 5.2). Malate shoot tissue was much higher than root tissue in both species ($p=0.015$).

Citrate concentrations were similar throughout the root system in *Brachiaria ruziziensis* under both in control and aluminium treatment (Figure 5.3). In *Brachiaria decumbens* the citrate concentration was highest in root tips in aluminium treated plants ($p=0.162$) but not in control plants (Figure 5.3). Shoot citrate

concentrations were higher than root concentrations in both species (p=0.003). Citrate shoot concentrations were significantly higher in *Brachiaria decumbens* than *Brachiaria ruziziensis* (p=0.008)

Phosphate concentration was similar throughout roots of *Brachiaria ruziziensis* under both control and aluminium conditions (Figure 5.4). In *Brachiaria decumbens* phosphate concentration was higher in the root tip than the more mature root in aluminium treated plants (p=0.201) but not in control plants (Figure 5.3).

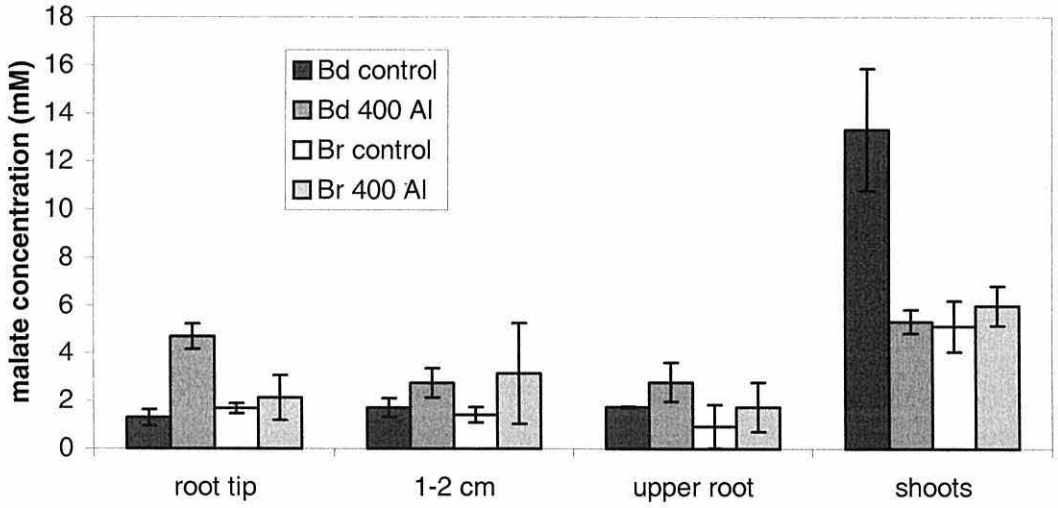

Figure 5.2: Relative malate concentration in root tip (0 to 1cm), elongation zone (1 to 2cm), mature upper roots (5 to 7cm from root tip) and leaf blades of *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) exposed to 0 or 400 μM aluminium. n=3.

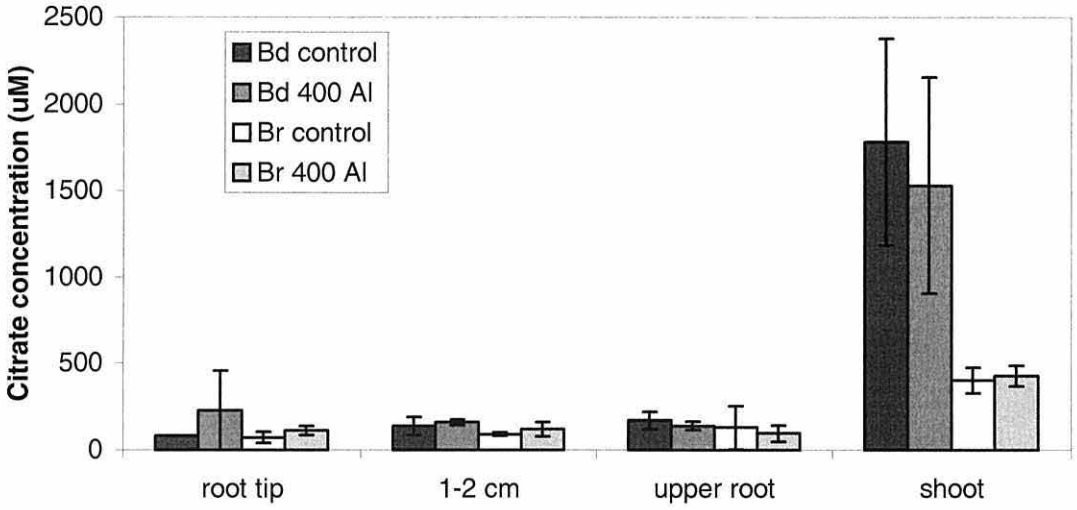

Figure 5.3: Relative citrate concentration in root tip (0 to 1cm), elongation zone (1 to 2cm), mature upper roots (5 to 7cm from root tip) and leaf blades of *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) exposed to 0 or 400 μM aluminium. n=3.

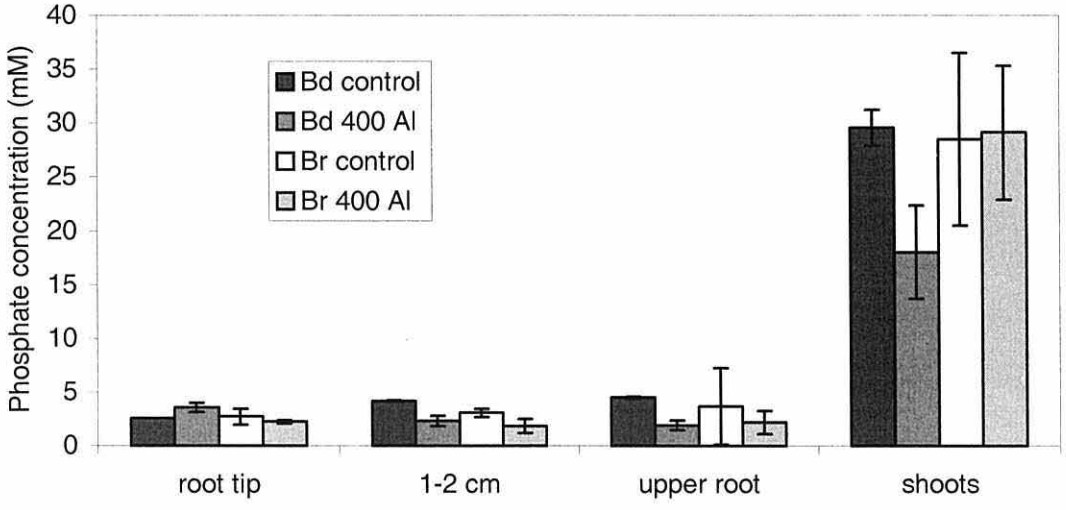

Figure 5.4: Spatial distribution of phosphate concentration in *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br) after treatment with or without 400 μM aluminium chloride.

5.4.2 Experiment Two: Phosphate starvation experiment

Experiment two, looking at the effect of phosphate starvation on organic acid root tip concentration, found that forty-eight hour phosphate starvation caused a doubling in malate concentration in root tips of *Brachiaria decumbens* in control conditions. There was also an increase in malate concentration in response to aluminium treatment. The combination of phosphate starvation and aluminium treatment caused a synergistic increase in malate concentration (p=0.171) (Figure 5.5). There was no effect of aluminium on malate concentration in the presence of phosphate. The presence or absence of phosphate had no effect on malate concentration in *Brachiaria ruziziensis* in control conditions. However a combination of phosphate starvation and aluminium treatment caused an increase in malate concentration (Figure 5.6).

Citrate root tip concentration was not affected by phosphate starvation in either *Brachiaria* species in control conditions (Figures 5.5 and 5.6). The effect of aluminium on citrate concentration was magnified in the absence of phosphate in both species (Bd p=0.179, Br p=0.221). In *Brachiaria ruziziensis* citrate and malate increase was only seen with a combination of phosphate starvation and aluminium treatment.

Fumarate concentrations were much lower than either malate or citrate concentrations in all treatment combinations. There was no effect of either phosphate starvation or aluminium treatment in *Brachiaria ruziziensis*, however, as for citrate and malate, the concentration of fumarate in *Brachiaria decumbens* was increased by a combination of phosphate starvation and aluminium treatment (0.178) (Figures 5.5 and 5.6).

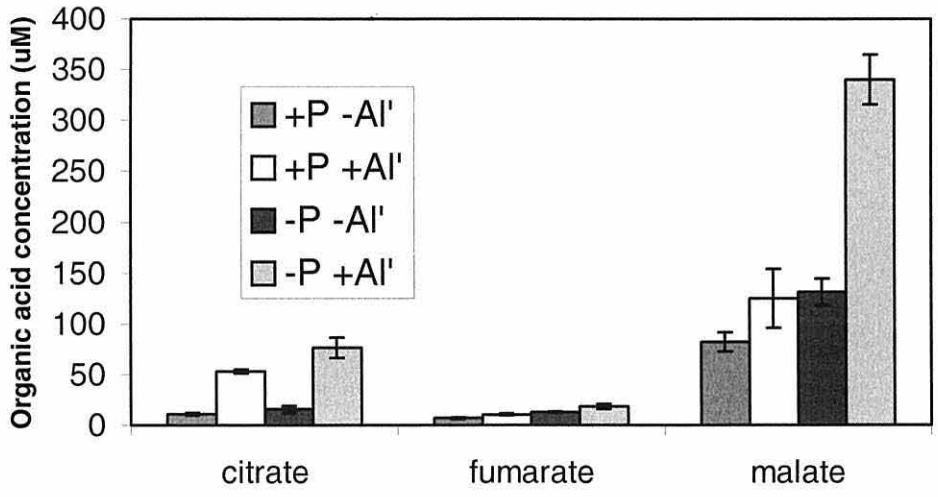

Figure 5.5: Effect of phosphate starvation (-P) and treatment with 0 (-Al) or 400 μM (+Al) aluminium treatment on citrate, fumarate and malate content of *Brachiaria decumbens* root tips. Organic acid root tip concentration in μM. ± S.E. n = 3.

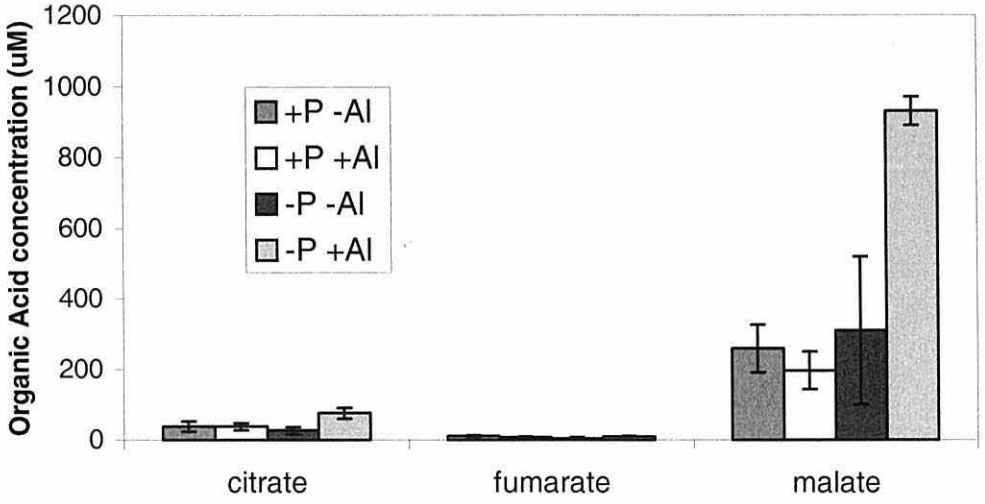

Figure 5.6: Effect of phosphate starvation (-P) and treatment with 0 (-Al) or 400 μM (+Al) aluminium treatment on citrate, fumarate and malate content of *Brachiaria ruziziensis* root tips. Organic acid root tip concentration in μM. ± S.E. n = 3.

Phosphate starvation caused a decrease in root tip phosphate concentration in both *Brachiaria* species (Figure 5.7). Aluminium treatment had no significant effect on phosphate root tip content in either *Brachiaria* species under either phosphate treatment (Figure 5.7).

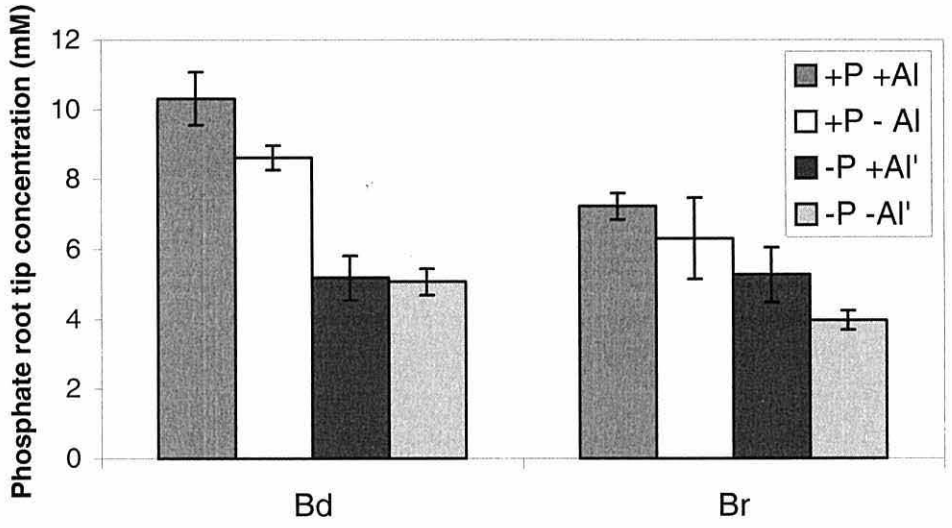

Figure 5.7: Effect of phosphate starvation (-P) and treatment with 0 (-Al) or 400 μM (+Al) aluminium treatment on phosphate root tip concentration (mM) in *Brachiaria decumbens* and *Brachiaria ruziziensis*. ± S.E. n=3.

5.3.3 Experiment Three: Aluminium dose response experiment

The third experiment investigated the effect of increasing aluminium treatment on root tip organic acid concentration. Treatment with aluminium caused a six-fold increase in citrate root tip concentration in both *Brachiaria decumbens* and *Brachiaria ruziziensis*, and a doubling of citrate concentration in *Triticum aestivum* (Figure 5.8). Citrate levels were approximately 8 times higher in the two *Brachiaria*

species than in *Triticum aestivum* (Figure 5.8), and approximately 100 μM higher in *Brachiaria decumbens* than *Brachiaria ruziziensis* at the 400 and 800 μM aluminium treatment levels with the addition of EDTA. Addition of EDTA caused a tripling in measurable citrate in all three species. Citrate concentrations were greater in *Brachiaria ruziziensis* than *Brachiaria decumbens* at lower aluminium treatments, with 50 μM aluminium causing near maximum citrate induction in *Brachiaria ruziziensis* but only 50% in *Brachiaria decumbens.* Maximum root tip citrate concentrations were reached after treatment with only 100 μM aluminium in all three species.

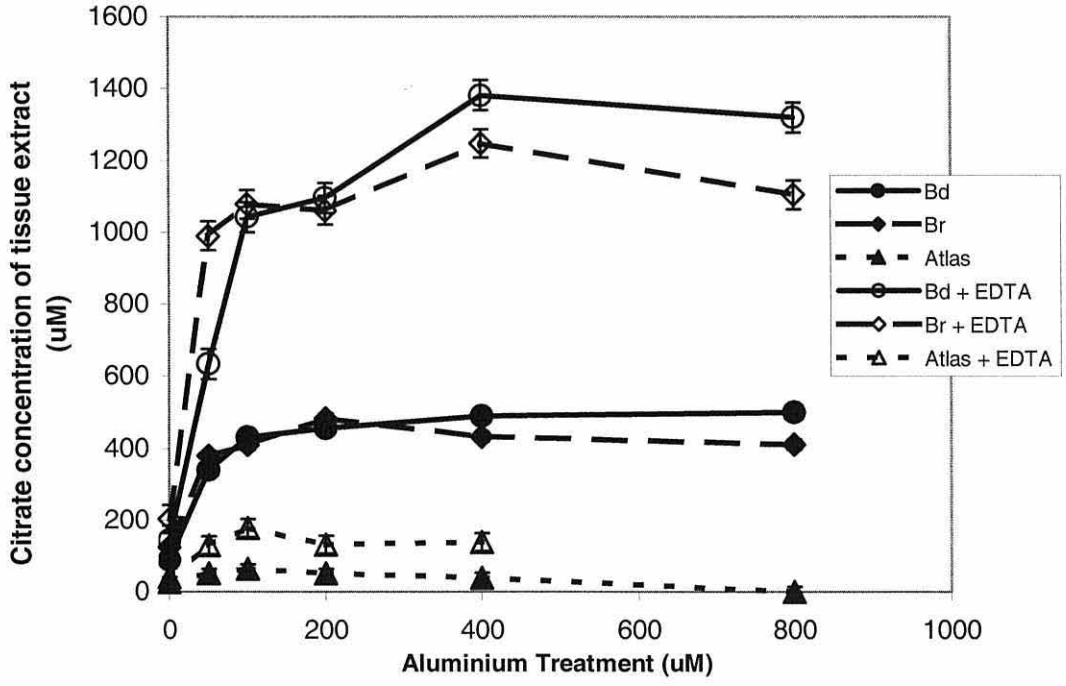

Figure 5.8: Effect of 0, 50, 100, 200, 400 and 800 μM aluminium treatment on citrate tissue content in *Brachiaria decumbens, Brachiaria ruziziensis* and *Triticum aestivum cv* Atlas, with and without the addition of 400 μM EDTA.

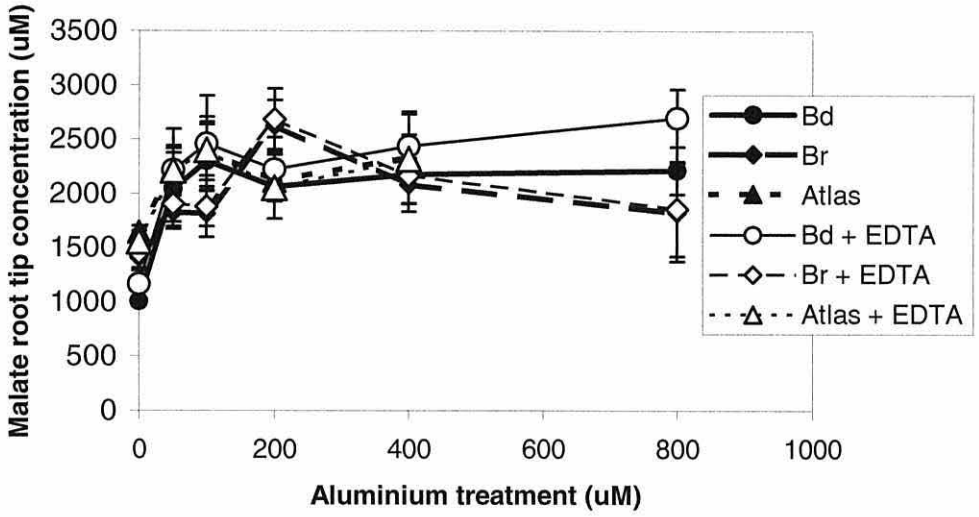

Figure 5.9: Effect of increasing aluminium treatment on malate tissue content in *Brachiaria decumbens, Brachiaria ruziziensis* and Atlas, with and without the addition of EDTA. ± S.E. n=3.

Aluminium treatment caused a doubling in malate root tip concentration in all three species (Figure 5.9). Malate concentration and response to aluminium treatment was not significantly different between the three species. Addition of EDTA did not significantly increase measurable malate concentrations. Malate concentrations were approximately twice as high as citrate concentrations in aluminium treated *Brachiaria* plants and 10 times as high as citrate in *Triticum aestivum*.

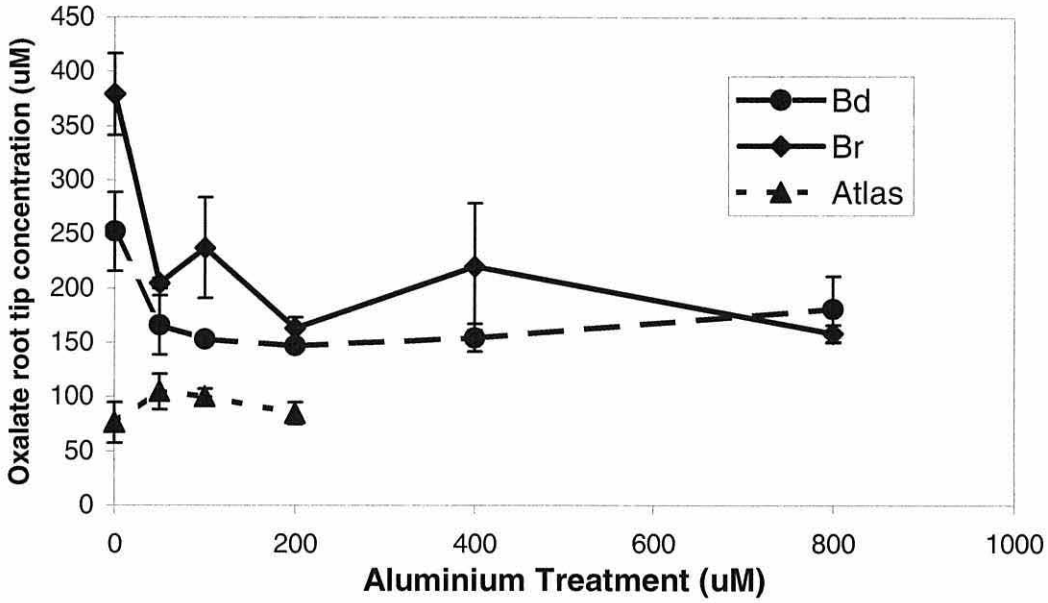

Figure 5.10: Effect of increasing aluminium treatment on oxalate root tip concentration (μM) in *Brachiaria decumbens, Brachiaria ruziziensis* and *Triticum aestivum cv* Atlas. All oxalate samples were with 400 μM EDTA. ± S.E. n = 3.

Oxalate root tip concentrations were 3 to 4 times higher in both *Brachiaria* species than in *Triticum aestivum* under control conditions, and approximately twice as high in aluminium treated plants (Figure 5.10). Aluminium caused a decrease in oxalate root tip concentrations in both *Brachiaria* species and had no significant effect on oxalate root concentrations in *Triticum aestivum* (Figure 5.10). Oxalate concentrations were significantly higher in *Brachiaria decumbens* than *Brachiaria ruziziensis* under control conditions, but were similar after treatment with aluminium. There was no data for oxalate concentrations without addition of EDTA. Oxalate concentrations were around the level of citrate under control conditions, and approximately one fifth of the malate concentrations.

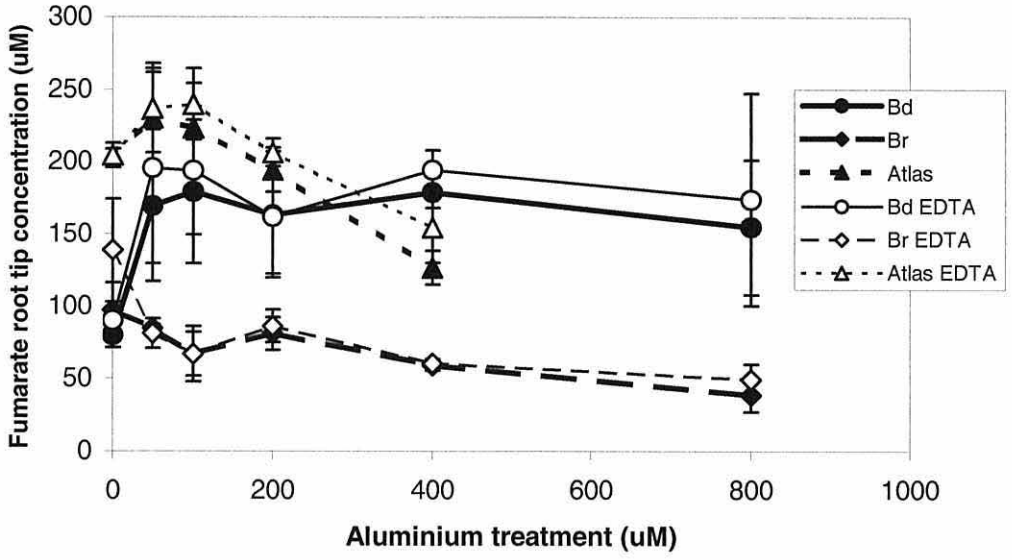

Figure 5.11: Effect of increasing aluminium treatment on fumarate root tip concentration (μM) in *Brachiaria decumbens, Brachiaria ruziziensis* and *Triticum aestivum cv* Atlas. With and without addition of 400 μM EDTA. ± S.E. n = 3.

Aluminium treatment caused an increase in fumarate root tip concentrations in *Brachiaria decumbens,* causing an approximate 2-fold increase (Figure 5.11). There was no response of fumarate concentration to aluminium in *Brachiaria ruziziensis* (Figure 5.11), and in *Triticum aestivum* aluminium caused a decrease. EDTA had no effect on measurable fumarate in any of the 3 species. Fumarate concentration was 2 to 3 times higher in *Triticum aestivum* than both *Brachiaria* species under control conditions

Fumarate concentration is almost double the oxalate concentration in *Triticum aestivum*, but similar in *Brachiaria decumbens* and significantly lower in *Brachiaria ruziziensis.*

Peak 3 represents a number of organic acids not separated by either of the carrier electrolytes used. Succinate and galactarate are thought to be two of the components of peak 3, although without full separation this cannot be confirmed. Aluminium caused a four-fold increase in peak 3 concentration in root tips of both *Brachiaria* species, but only a doubling in *Triticum aestivum* (Figure 5.12). There was no

significant difference in peak 3 concentration or response to aluminium between the two *Brachiaria* species. EDTA had no significant effect on measurable peak 3 concentration.

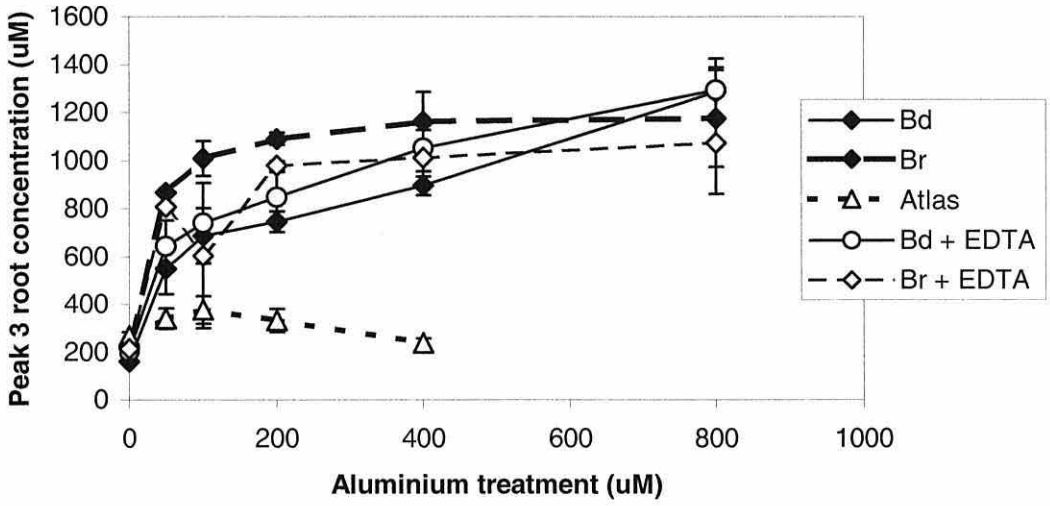

Figure 5.12: Effect of increasing aluminium treatment on peak 3 (a combination of several unidentified organic acids) tissue content in *Brachiaria decumbens, Brachiaria ruziziensis* and *Triticum aestivum*. With and without 400 µM EDTA in both *Brachiaria* species. ± S.E. n = 3.

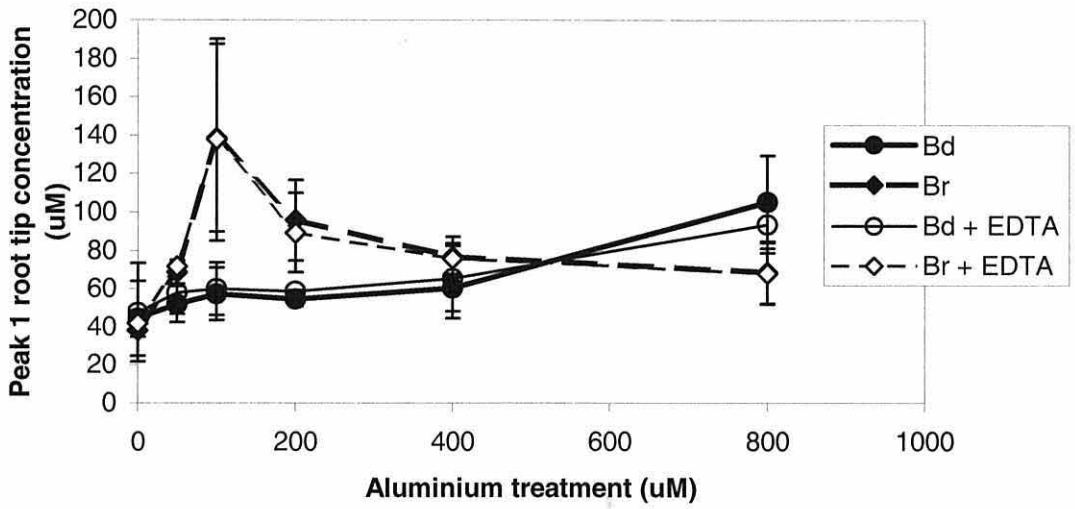

Figure 5.13: Effect of increasing aluminium treatment on peak 1 tissue content in *Brachiaria decumbens* and *Brachiaria ruziziensis*. With or without 400 μM EDTA. ± S.E. n = 3.

Peak 1 is as yet unidentified and did not correspond to any of the common organic acids tested such as malonic acid, maleic acid, formate, acetate or pyruvate, it was also not detected in *Triticum aestivum.* Peak 1 did not show a significant aluminium effect in *Brachiaria decumbens,* but did show a 2 to 3 fold increase in concentration in *Brachiaria ruziziensis* in response to 100 μM aluminium (Figure 5.13). EDTA had no effect on Peak 1 concentration.

The two root morphologies did not have significantly different concentrations of citrate, fumarate, malate, phosphate or the Peak 3 aggregate in the root tips (Figure 5.14). However the thick roots had significantly higher concentrations of Peak 1 (completely unidentified anion) than found in normal root type (Figure 5.14).

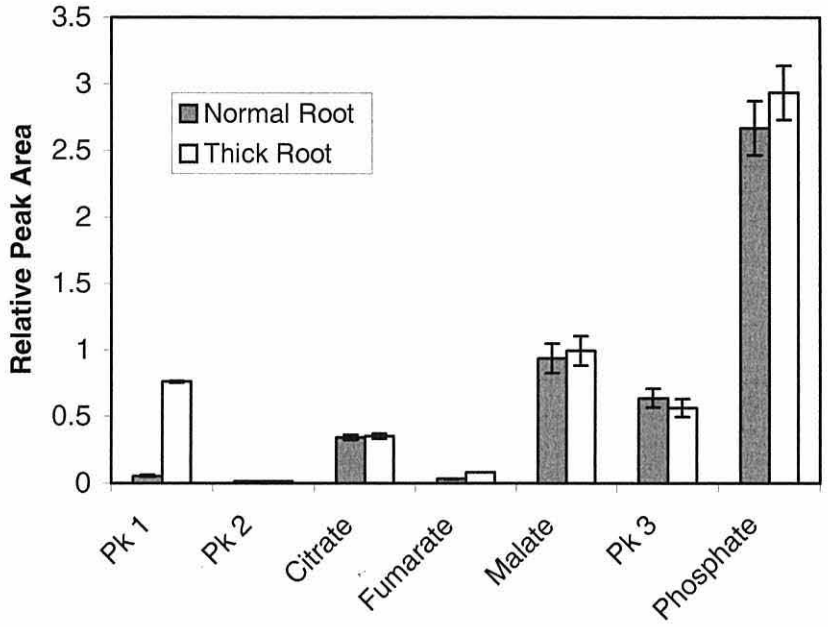

Figure 5.14: Organic acid comparison for the two different root morphologies shown in *Brachiaria decumbens*. ± S.E. n = 3

5.3.4 Experiment Four: Organic acid time response experiment

There was an initial increase in citrate concentration in both control and aluminium treatments in *Brachiaria decumbens.* Citrate concentrations then decreased to a base level within four hours. There was no initial increase in citrate concentration in *Brachiaria ruziziensis.* Aluminium treatment caused a sustained increase in citrate concentration after four hours in *Brachiaria ruziziensis* and after ten hours in *Brachiaria decumbens* (Figure 5.15). The citrate concentration was maintained at an elevated level for remainder of experiment (72 hours). A large increase in citrate level was seen in control treatments between 24 and 72 hours in both species, although the induction in control treatment was not as great as caused by aluminium treatment.

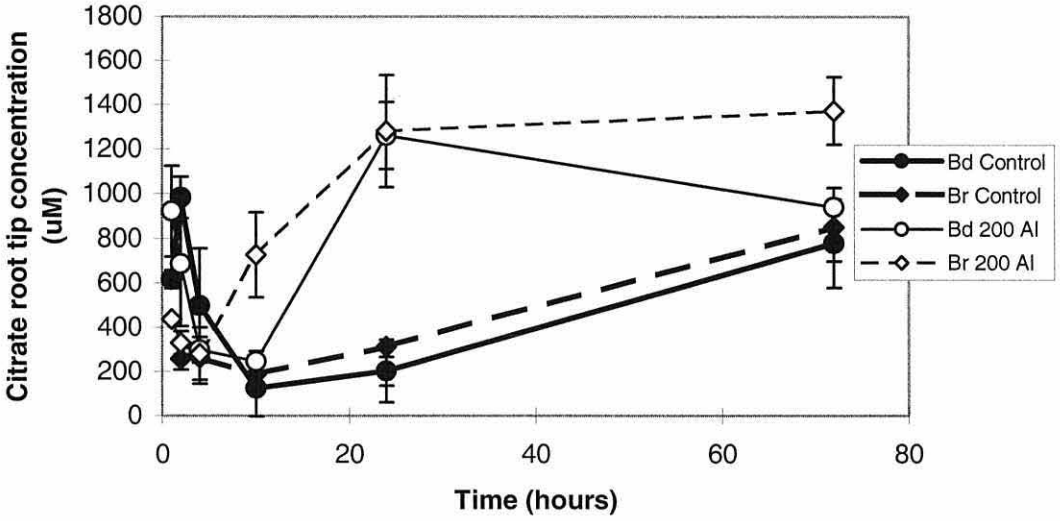

Figure 5.15: *Brachiaria decumbens* and *Brachiaria ruziziensis* citrate root tip concentration (μM) after 1, 2. 4, 10, 24 and 72 hour treatment with 0 or 200 μM aluminium. With addition of 400 μM EDTA. ± S.E. n = 3.

Malate increased in response to aluminium treatment after 4 hours in *Brachiaria ruziziensis* and after 10 hours in *Brachiaria decumbens* relative to control levels (Figure 5.16). The increase in malate root tip concentration in response to aluminium was relatively small in comparison to the increase seen in the control treatments after 24 hours in both species. By 72 hours malate concentration was 5 times higher in control than aluminium treatments. As with citrate concentration, there was an initial elevation in malate concentration in *Brachiaria decumbens* in both control and aluminium treatments. This rapidly decreased to base levels by 4 hours.

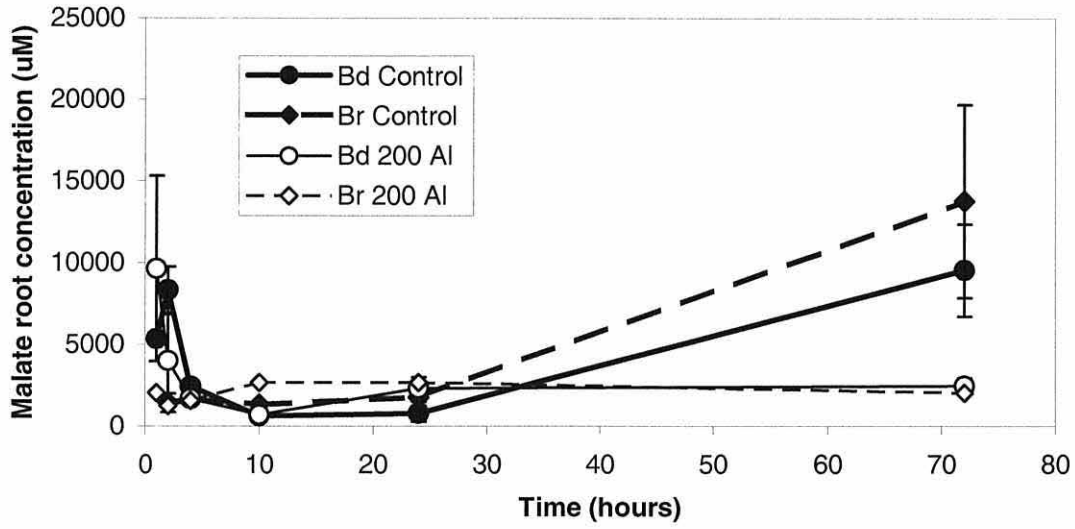

Figure 5.16: *Brachiaria decumbens* and *Brachiaria ruziziensis* malate root tip concentration (μM) after 1, 2. 4, 10, 24 and 72 hour treatment with 0 or 200 μM aluminium. With addition of 400μM EDTA. ± S.E. n = 3.

Fumarate followed a similar pattern to malate response. Aluminium caused a doubling in fumarate concentration after 4 hours in *Brachiaria ruziziensis* and after 10 hours in *Brachiaria decumbens* (Figure 5.17), while concentrations remained static under control conditions. A 3-fold increase in fumarate concentration was seen in control treatments between 24 and 72 hours, with fumarate concentrations higher in control treatments than aluminium treatments after 72 hours. Again there was an initial elevated fumarate concentration in *Brachiaria decumbens* under both control and aluminium treatment, which decreased to base levels by 4 hours.

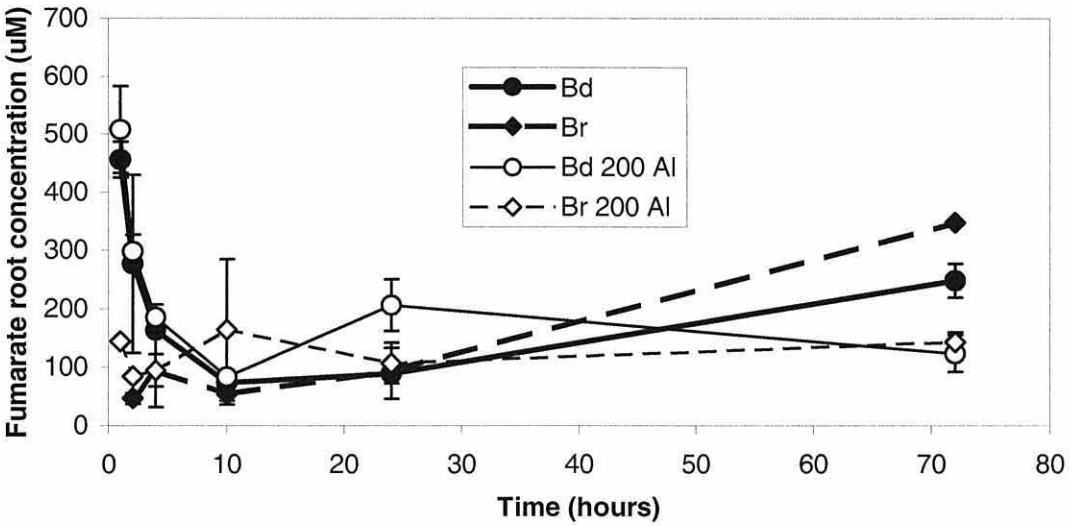

Figure 5.17: *Brachiaria decumbens* and *Brachiaria ruziziensis* fumarate root tip concentration (μM) after 1, 2. 4, 10, 24 and 72 hour treatment with 0 or 200 μM aluminium. With addition of 400 μM EDTA. ± S.E. n = 3.

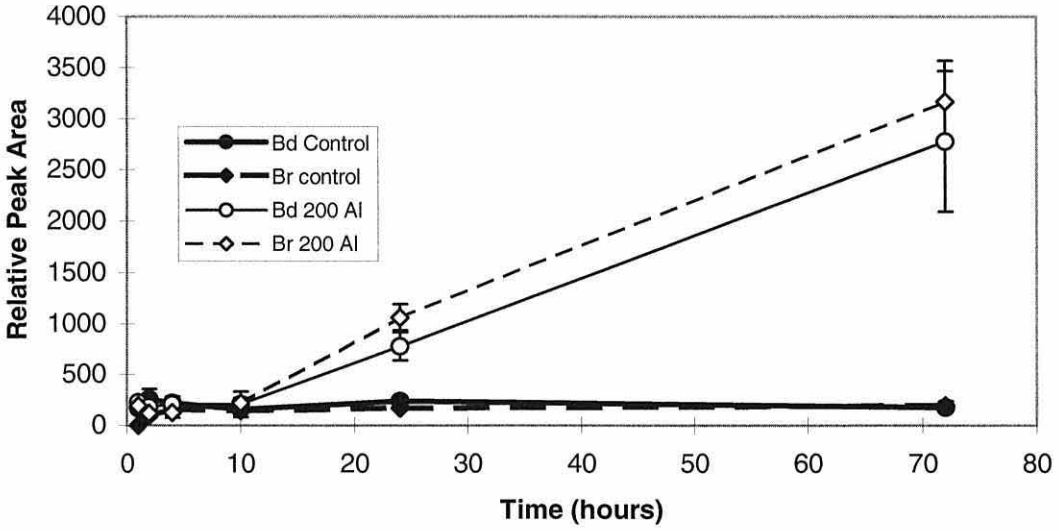

Figure 5.18: *Brachiaria decumbens* and *Brachiaria ruziziensis* peak 3 (succinate/galactarate) root tip concentration (μM) after 1, 2. 4, 10, 24 and 72 hour treatment with 0 or 200 μM aluminium. With addition of 400 μM EDTA. ± S.E. n=3.

Peak 3, a mixture of un-separated organic acids including succinate, did not show the increase in concentration over time in control treatments as seen in citrate, malate and fumarate (Figure 5.18). Aluminium caused a large sustained increase in peak 3 concentration after 10 hours in both *Brachiaria* species.

Peak 1 (unidentified anion) concentration increased in control treatments of both species after 24 hours with no corresponding increase in aluminium treated plants (Figure 5.19).

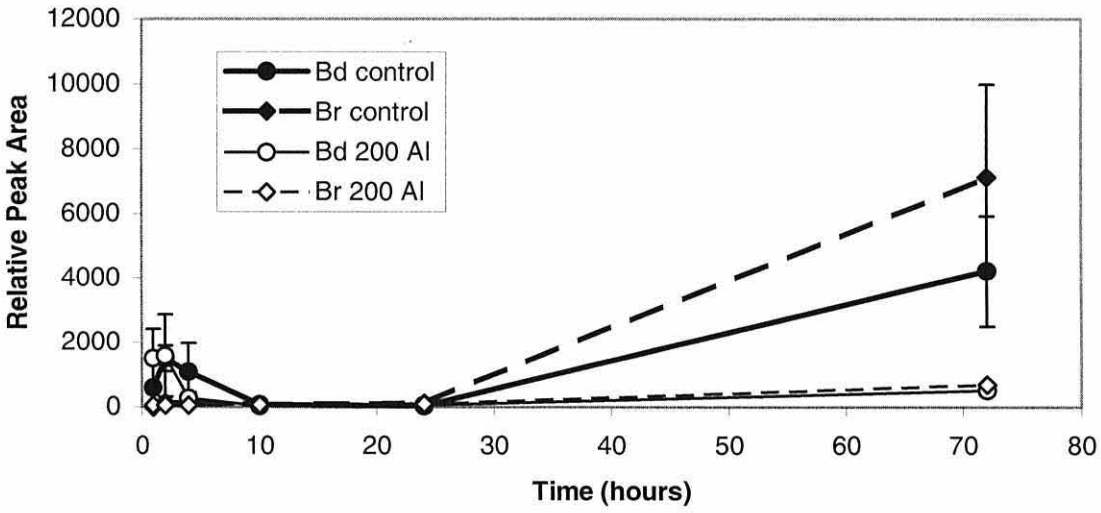

Figure 5.19: *Brachiaria decumbens* and *Brachiaria ruziziensis* peak 1 (unidentified) root tip concentration (μM) after 1, 2. 4, 10, 24 and 72 hour treatment with 0 or 200 μM aluminium. With addition of 400 μM EDTA. ± S.E. n = 3.

5.3.5 Experiment Five: Apoplastic or symplastic aluminium

As citrate is the only organic acid which changed concentration significantly with the addition of EDTA, the concentration of citrate released by addition of EDTA can be used to estimate relative aluminium partitioning between the apoplast and symplast. Washing roots with phosphate caused a 33% reduction in measurable citrate in EDTA treated samples (Figure 5.20), but had no effect on samples containing no EDTA. Assuming that washing roots with phosphate solution removed all exchangeable aluminium from the apoplast, it is possible to estimate approximate apoplastic and symplastic aluminium partitioning between the symplast and apoplast. Aluminium bound citrate is calculated by subtracting the concentration of none EDTA treated sample from EDTA treated sample. The difference between the phosphate washed EDTA treated sample and the distilled water washed EDTA treated sample represents the bound to exchangeable apoplastic aluminium. The remainder of the citrate bound aluminium must therefore be symplastic in origin It was estimated that of the aluminium bound to citrate approximately 44% was apoplastic in origin (Table 5.1).

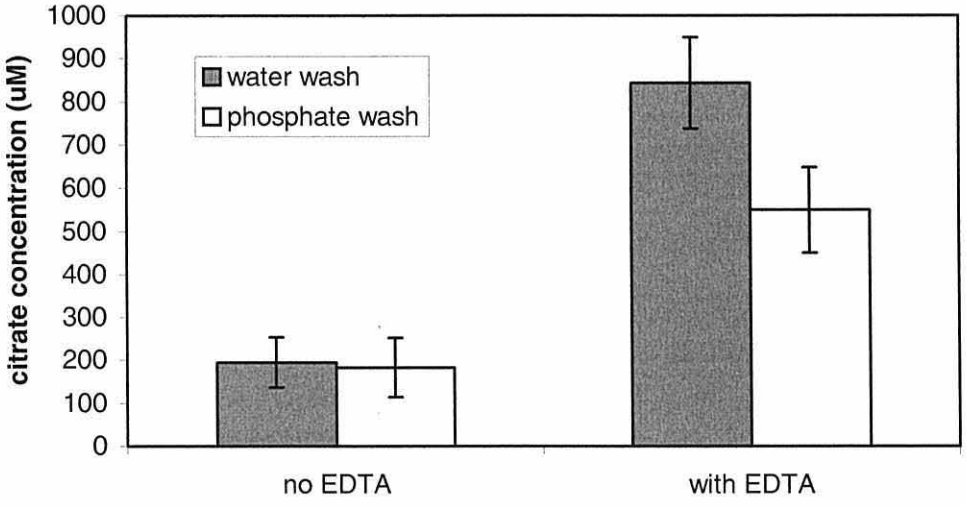

Figure 5.20: Citrate root tip concentration in *Brachiaria decumbens* treated with 400 μM aluminium, roots washed with either distilled water or 800 μM phosphate and with or without addition of EDTA. ± S.E. n = 3.

Table 5.1: Partitioning of aluminium citrate (μM) based on data from Figure 5.19.

Unbound Citrate	Citrate bound to apoplastic aluminium	Citrate bound to symplastic aluminium
195 μM	293 μM	365 μM

5.3.6 Experiment Six: Measurement of exudation

5.3.6.1 Time response experiment

Examination of a 1 ml sample of the bulk nutrient solution surrounding the roots showed that only two organic acids were detectable in the exudates from *Brachiaria ruziziensis*. These were malate and oxalate. Despite the large induction of citrate in tissue samples in response to aluminium, no citrate was detected in the exudate even with additions of 1 and 2 mM EDTA, which would in theory release any citrate bound to aluminium and other cations.

Malate exudation was measurable after 6 hours in *Brachiaria ruziziensis* under control and all aluminium treatments (Figure 5.21). Malate exudation increased with increasing aluminium treatment ($p=0.036$).

Oxalate concentrations in the exudates were much lower by a factor of 10 than malate (Figure 5.22). Oxalate was only detectable in exudate of *Brachiaria ruziziensis* plants treated with aluminium, and not in exudates from control treatments. As with malate oxalate was first detectable 6 hours after start of treatment and increasing aluminium treatment caused increasing oxalate exudation.

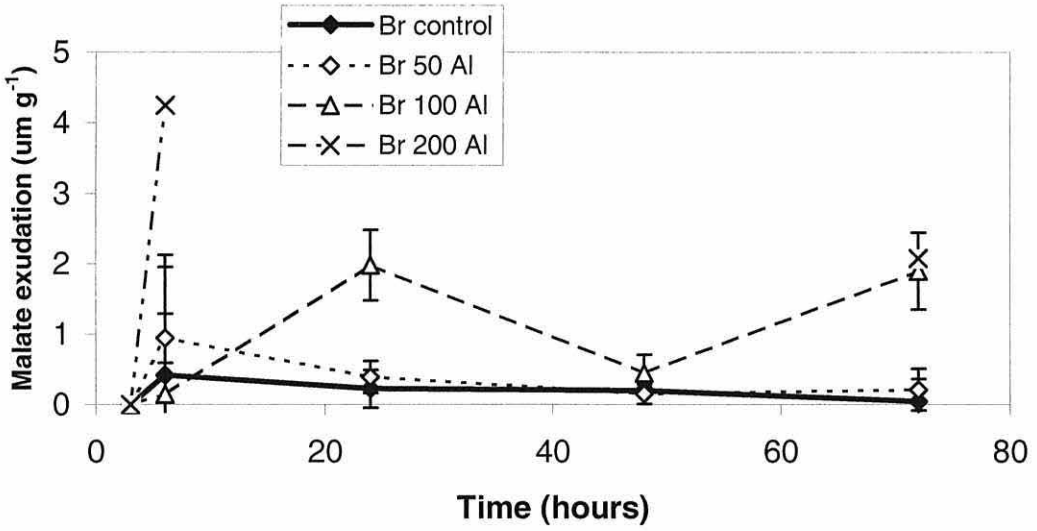

Figure 5.21: Malate exudation (μM g^{-1}) time course from roots of *Brachiaria ruziziensis* treated with 0, 50, 100 and 200 μM aluminium in 100 μM $CaCl_2$ solution ± S.E. n = 3.

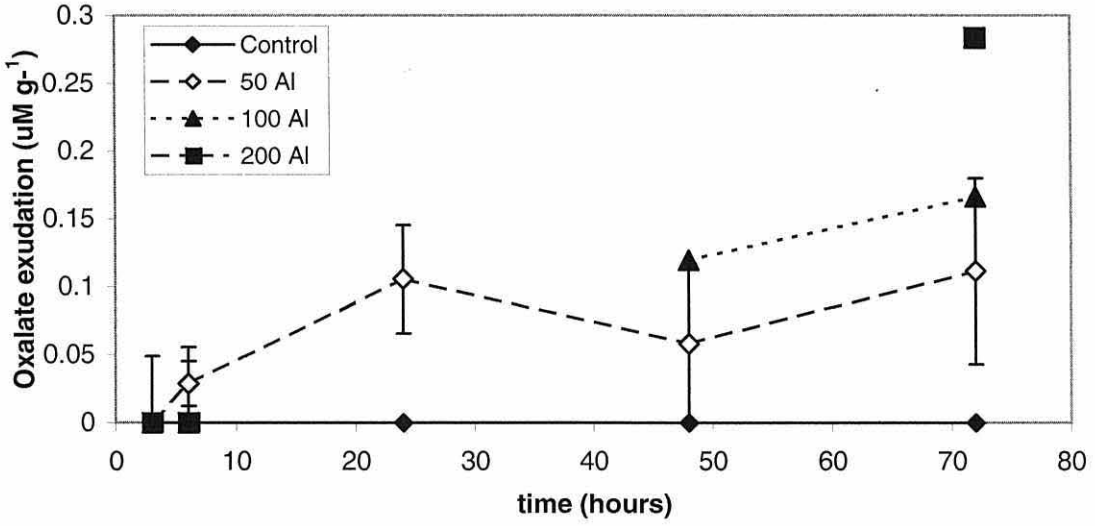

Figure 5.22: Oxalate exudation (μM g^{-1}) time course from roots of *Brachiaria ruziziensis* treated with 0, 50, 100 and 200 μM aluminium in 100 μM $CaCl_2$ solution ± S.E. n=3.

5.3.6.2 Pico sampling

Exudate samples were taken from the nutrient solution at boundary layer between the surface of the root 3mm back from the root cap, and the nutrient solution in *Brachiaria decumbens.* Only malate was detectable in the exudates. Oxalate may have been at detectable concentrations, however due to the electrophoretic system used, the oxalate peak was not separated from the inorganic cations. Citrate was not detected, however this experiment was carried out prior to development of the organic acid protocol, therefore there was no use of EDTA (which was used to release citrate bound to aluminium in other experiments) and tungstate was used as internal standard instead of molybdate.

Measurable malate exudation in *Brachiaria decumbens* was higher in control plants than aluminium treated plants (Figure 5.23). The exudate measurements from the *Brachiaria decumbens* root surface were 20 times higher than *Brachiaria ruziziensis* exudate measurements from the bulk nutrient solution.

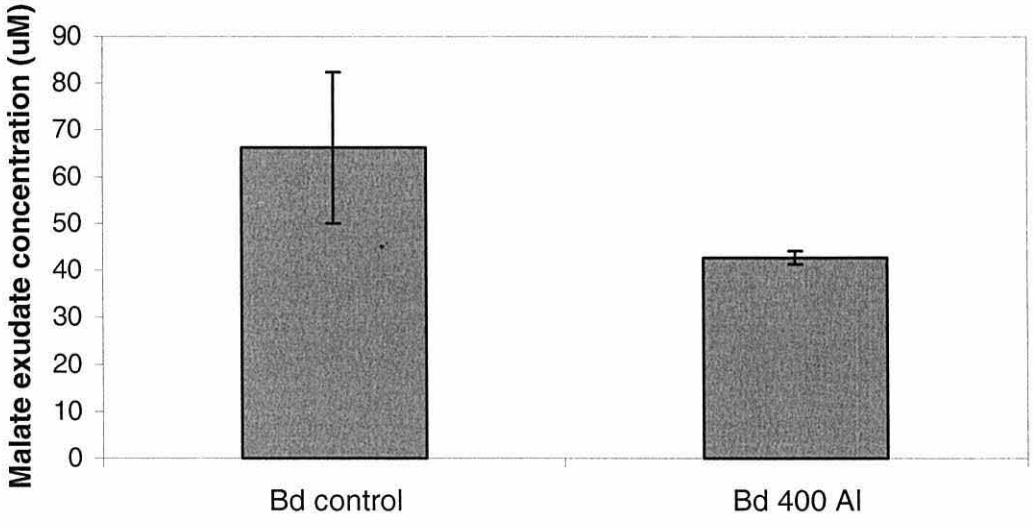

Figure 5.23: Malate exudation (μM) from the root tip surface of *Brachiaria decumbens* after treatment with 0 or 400 μM aluminium in 200 μM $CaCl_2$. ± S.E. n=2.

5.4 Discussion

5.4.1 Experiment One: Spatial distribution of organic acids

Organic exudation in response to aluminium is thought to be highest at the root tip than in mature root tissue (Kochian, 1995; Jones and Darrah, 1995), this reflects the major the fact that aluminium primarily affects tissue undergoing rapid growth and division such as root apices (Ryan *et al.*, 1993). The results for *Brachiaria decumbens* generally reflect this idea of increased organic acid concentration at the root tip in response to aluminium, however, the trend is much less distinct in *Brachiaria ruziziensis.*

5.4.2 Experiment Two: Phosphate starvation experiment

In *Brachiaria decumbens* malate concentration was found to double in response to 48-hour phosphate starvation and there was also a less pronounced but significant increase in citrate concentration. Phosphate starvation or deficiency has been shown to induce organic acid accumulation and exudation in a number of species including *Brassica* species, *Lupinus albus* (Gardner *et al.*, 1983), alfalfa (Lipton *et al.*, 1987) and *Brassica napus* (Hoffland *et al.,* 1989) to enable increased phosphate scavenging. However although this has been investigated in graminoid species, it has not been shown to occur. The doubling in malate concentration in *Brachiaria decumbens* suggests that the better acid soil adapted species *Brachiaria decumbens* may employ the organic acid phosphate scavenging mechanism to a certain degree. However increased organic acid tissue content does not necessarily correlate with increased exudation. The sampling method used in this experiment includes mainly vacuolar as well as cytosolic organic acids. The measured increase in malate may therefore reflect an increase in vacuolar concentration rather than cytosolic concentration, when it is cytosolic concentration that is important for driving exudation. The lack of response in *Brachiaria ruziziensis* implies that organic acids are not likely to be used to increase phosphate uptake in this species.

Although the evidence for the operation of an organic acid phosphate scavenging mechanism may not be conclusive, phosphate starvation appears at first glance to have a significant effect on organic acid induction in response to aluminium treatment in *Brachiaria decumbens* and *Brachiaria ruziziensis*. Phosphate starvation increased the concentration of malate induced in response to aluminium in both species. A similar trend was seen for citrate concentration in *Brachiaria decumbens* but not in *Brachiaria ruziziensis*. However the increased induction of malate and citrate to aluminium treatment in phosphate-starved plants could be due to aluminium solution chemistry. In the nutrient solution containing phosphate, some of the aluminium will bind to the phosphate, reducing the concentration of aluminium available to the plant in comparison to the nutrient solution containing no phosphate. However from the dose response data (Experiment 3) increasing the aluminium concentration from 200μM aluminium (used in the phosphate starvation experiment) did not further increase citrate or malate root tip concentrations to any great degree. Therefore a small increase in aluminium at the aluminium treatment levels used in the phosphate starvation experiment would not be expected to cause such a large increase in malate or citrate concentration. It can therefore be concluded that the higher induction of citrate and malate under phosphate starvation in response to aluminium may in part be due to an effect of phosphate starvation.

5.4.3 Experiment Three: Aluminium dose response experiment

The more aluminium tolerant *Brachiaria* species generally have higher organic acid concentrations than the aluminium sensitive *Triticum aestivum*, although malate root tip concentrations are similar in all three species and *Triticum aestivum* had significantly higher fumarate concentrations than *Brachiaria ruziziensis*. Citrate and Peak 3 concentrations are particularly different in both *Brachiaria* species compared to *Triticum aestivum*, with much higher concentrations in the two *Brachiaria* species. The higher citrate, Peak 3 and oxalate levels in the *Brachiaria* species may well go some way to explain their greater aluminium tolerance over *Triticum aestivum*. The higher citrate, Peak 3 and oxalate concentrations in *Brachiaria decumbens* and *Brachiaria ruziziensis* would enable the two *Brachiaria* species to accumulate higher

internal aluminium concentrations than *Triticum aestivum* without aluminium becoming toxic to the cells.

There was little difference in organic acid response in the root tip tissue to increasing aluminium treatment between *Brachiaria decumbens* and *Brachiaria ruziziensis*. Malate response was identical, however citrate and peak 3 were induced to a greater degree at lower aluminium treatments (50 and 100 μM aluminium) in *Brachiaria ruziziensis* than *Brachiaria decumbens*. *Brachiaria ruziziensis* had lower fumarate and lower oxalate concentrations, particularly in control plants, than *B decumbens*. However fumarate and oxalate were at relatively low concentrations compared to citrate and malate so would have only a small impact on the internal detoxification capacity.

This agrees with the EDTA data. Addition of EDTA, which has a very high cation binding co-efficient, releases organic acids bound to aluminium and other cations. Treatment of the extracted samples with EDTA caused a large increase in the measurable citrate in the aluminium treated samples (but not control samples), but had no significant effect on measurable malate, fumarate or Peak 3 concentrations in either control or aluminium treated samples. This indicates that citrate is more important than malate, fumarate or Peak 3 (which includes succinate) for binding aluminium (forming Al-citrate) and so is probably the organic acid mainly responsible for internal aluminium sequestering in *Brachiaria* root tip cells, even though malate is at much higher concentrations in the roots.

Further evidence for the importance of citrate comes from the large induction of citrate in response to aluminium, which increased citrate concentration from the μM level of fumarate and oxalate to much nearer the mM level of malate.

The much greater concentration of citrate in the two *Brachiaria* species than *Triticum aestivum* may partly explain the much greater aluminium tolerance of the *Brachiaria* species in relation to *Triticum aestivum*. This greater citrate

concentration would enable *Brachiaria* to accumulate and tolerate much higher internal concentrations of aluminium than *Triticum aestivum.* This increase in tissue concentration does not necessarily mean that there is a similar induction of citrate exudation.

Oxalate concentrations decreased with aluminium treatment in the two *Brachiaria* species. This may be caused by increased exudation of oxalate. Concentrations in root tips of *Triticum* increased slightly with aluminium treatment, however the oxalate levels in the two *Brachiaria* species were significantly higher than in *Triticum aestivum.* Peak 3 includes succinate and other (unidentified due to poor separation) anions However, they show a very strong increase in response to aluminium treatment in both *Brachiaria* species. There was a significantly higher increase in *Brachiaria ruziziensis* than *Brachiaria decumbens,* particularly over the lower aluminium treatments.

The two root morphologies showed almost identical organic acid content except for Peak 1 (unidentified anion), which showed much higher levels in the thick root type. Changes in root morphology including altered branching patterns, absence or shortening of root hairs and less root death can occur in plants grown in hydroponic culture compared to soil grown plants. Hydroponic plants also have plenty of water, no physical barriers to growth, and a completely different nutrient supply and chemistry than soil grown plants. Whether this differing root morphology is an artefact of hydroponic growth conditions, or whether it is produced in response to some stress such as nutrient deficiency or toxicity is unknown. However the thick root type was found to be equally susceptible to aluminium toxicity in terms of reduced growth rate, and showed no difference in induction of organic acids such as malate, citrate, oxalate and fumarate than the finer roots, and so is probably not connected to aluminium tolerance in *Brachiaria decumbens* or *Brachiaria ruziziensis.*

The elevated Peak 1 concentration at 100 μM aluminium treatment in *Brachiaria ruziziensis* could be explained by contamination of the sample with tissue from the thick root type, which has elevated levels of Peak 1 compared to the finer roots.

5.4.4 Experiment Four: Organic acid time response experiment

Induction of citrate, malate and fumarate in response to aluminium treatment occurs faster in *Brachiaria ruziziensis* (after 4 hours) than in *Brachiaria decumbens* (after 10 hours). The increase in citrate is again large, as found in the aluminium dose response experiments. The initial elevated citrate concentration in both control and aluminium treatments is probably caused by nutrient stress prior to experimental treatment – the nutrient solution was changed every 2 days). Malate, fumarate, citrate and Peak 1 in both *Brachiaria ruziziensis* and *Brachiaria decumbens* increased in the control treatment between 24 and 72 hours. This is probably also due to nutrient stress. The increase in malate, fumarate and Peak 1 concentrations in response to nutrient stress is much larger than their response to aluminium treatment. Citrate is the exception, where aluminium stress caused as great an increase in citrate concentration as nutrient stress. This could be due to citrate being more important in terms of aluminium tolerance, whereas malate and fumarate are more important in response to nutrient stress. However, measurement of organic acid accumulation in tissue does not always relate to increased organic acid production. Malate production may be just as high under aluminium stress as under nutrient stress but due to greater exudation rates the tissue concentration is not able to build up to such a great extent.

Interestingly, aluminium treatment prevents the increases in malate, fumarate and Peak 1 concentrations in response to nutrient stress that are seen in control plants. This could be due to greater exudation of these organic acids under aluminium stress, which may decrease their accumulation in the root tips.

The huge increase in citrate, malate, fumarate and Peak 1 in response to nutrient stress could mean that these organic acids are important in nutrient acquisition in

nutrient-limiting situations. The role of organic acids in nutrient acquisition of phosphate and iron has been shown for a number of species. *Brachiaria decumbens* and *B. ruziziensis* do not appear to increase organic acid exudation in response to phosphate starvation, and as graminoid species they will use Strategy II iron acquisition which does not involve increased organic acid exudation. This would suggest that the nutrient stress response in *Brachiaria* is not due to either phosphate or iron, but another nutrient or combination of a number of nutrients.

The increase in citrate, malate and fumarate root tip tissue concentrations in response to nutrient stress would increase the negativity of the cytosol, increasing the electropotential gradient across the cell membrane, and so increase the uptake of positive nutrients such as calcium, magnesium and potassium.

Experiments on *Fagopyrum esculentum* (buckwheat) have shown a rapid induction of organic acid exudation, taking as little as 30 minutes (Ma *et al.*, 1997b, Zheng *et al.*, 1998b). The 4 to 10 hours taken by both *Brachiaria* species appears slow. However the measurements made here are on tissue concentration, not exudation. Increased induction of exudation in *Brachiaria* may well be much more rapid, involving the increased transport through specific anion channel. If increased exudation is reliant on increased transport via anion channels then there would not necessarily be an increase in bulk tissue concentrations.

The organic acid concentrations were almost identical between the two *Brachiaria* species whatever the aluminium treatment. This suggests that organic acid tissue concentration is not responsible for their different aluminium tolerances. The greater aluminium tolerance in *Brachiaria decumbens* could be due to greater exudation rates than *Brachiaria ruziziensis*. If *Brachiaria decumbens* had a higher concentration of anion channels but similar cytosolic organic acid concentrations then the exudation rate would be greater.

5.4.5 Experiment Five: Symplastic or apoplastic aluminium?

Addition of EDTA hugely increased measurable citrate during the previous experiments in this chapter. Only a small increase in citrate was seen in non-aluminium treated samples, leading to the conclusion that citrate was bound predominantly to aluminium. However the aluminium bound to the citrate could be from the cell interior, sequestered in the vacuole or cytoplasm, or it could be from the apoplast, binding to the symplastic citrate as the citrate was forced through the cell walls and apoplast during the sample extraction process.

By washing roots with phosphate solution would in theory remove loosely bound aluminium from the apoplast. Not all apoplastic aluminium would be removed by this process, particularly aluminium tightly bound to cation exchange sites. By comparing the aluminium bound citrate from roots washed with distilled water and those washed with phosphate solution a rough and ready estimate of symplastic relative to apoplastic aluminium can be measured.

The data showed a surprisingly high level of symplastic aluminium with 56% of the aluminium bound to citrate coming from the cell interior. However this will be an over-estimate as the phosphate wash will not remove all apoplastic aluminium, so that there will therefore be a bias towards symplastic estimate. Even so, it indicates that a considerable proportion of the root tip aluminium measured in Chapter Four is symplastic in origin and that both *Brachiaria* species are accumulating relatively high concentrations of aluminium in roots. This aluminium accumulation will require a high detoxifying capacity, which appears to be primarily met by citrate induction in both *Brachiaria* species.

5.4.6 Experiment Six: Measurement of exudation

Exudation of a variety of organic acids has been proposed as one of the most important mechanisms of aluminium tolerance in a number of plant species (e.g. Ma *et al.* 1997a, 1997b, 1997c; Delhaize *et al.*, 1993b). The organic acids bind to aluminium, detoxifying it and excluding it from uptake by plants, where it could

damage cell components. Different plant species have been shown to have different organic acid exudate compositions. Some, such as wheat, predominantly exude malate (Delhaize *et al.*, 1993b). Other species such as *Cassia tora* use citrate (Ma *et al,* 1997c), and some such as *Fagopyrum esculentum* (buckwheat) use oxalate (Ma *et al.,* 1998; Zheng *et al.*, 1998). The composition of exudates may also change over time, with specific organic acids increasing or decreasing as the aluminium exposure continues.

In both *Brachiaria decumbens* and *Brachiaria ruziziensis* the major organic acid component of the exudates was found to be malate. This was not surprising considering that malate was also the most abundant organic acid in root tip tissue. However what was surprising was that the second most abundant organic acid in root tip tissue, citrate, was not detectable in the exudates even after aluminium treatment. This does not mean that citrate was not exuded, as the levels may have been too low to detect, but very low levels indicate that citrate does not play an important role in excluding aluminium from root tips of *Brachiaria decumbens* and *Brachiaria ruziziensis.*

Equally as interesting was the detection of oxalate in exudates of aluminium treated plants. Oxalate is almost certainly present in exudates of control plants but at a very low level. This indicates a massive increase in oxalate exudation in response to aluminium in *Brachiaria ruziziensis.* This is interesting because oxalate is the most efficient chelator of aluminium of all the organic acids (Ginting *et al.*, 1998), and is often the dominant organic acid exuded from very aluminium tolerant species, such as *Fagopyrum esculentum,* in response to aluminium (Ma *et al.,* 1997; Zheng *et al.,* 1998).

Oxalate was at a much lower tissue concentration than citrate by a factor of ten. It would therefore be expected that citrate would be at a much higher concentration in the exudates than oxalate, but this was not the case. This could be because the large tissue citrate concentration is mainly vacuolar in origin, with much lower cytosolic

concentrations than oxalate. This would fit the EDTA data, which showed that nearly half the citrate was bound to aluminium inside the cell where it would most likely be transported to the vacuole for storage away from potentially sensitive cell components. This would still leave a large concentration of citrate not bound to aluminium acting as latent internal aluminium detoxification capacity. To be effective in binding any further aluminium, the remaining citrate would have to be in the cytosol to bind aluminium entering the cell. If this is the case and there are still relatively high citrate levels in the cytoplasm, then the root tip cells must be preferentially exporting oxalate and not citrate.

The exudation of high concentrations of oxalate in response to aluminium would explain the decrease seen in oxalate tissue concentrations with increasing aluminium treatment in both *Brachiaria* species. Assuming that the decrease in oxalate concentration in root tips in both *Brachiaria* species is due to increased exudation in response to aluminium, then the higher oxalate concentration in *Brachiaria decumbens* control plants and the consequent larger decrease in root tip oxalate concentration in response to aluminium in *Brachiaria decumbens* than *Brachiaria ruziziensis,* would suggest that *Brachiaria decumbens* may be exporting higher concentrations of oxalate in its exudates than *Brachiaria ruziziensis.* However, in the absence of oxalate exudate data for *Brachiaria decumbens* this is just supposition.

If malate is exuded in response to aluminium treatment, this may explain why malate tissue concentration did not increase greatly compared to the rise seen in response to nutrient stress. This assumes that exudation in the control plants under nutrient stress did not increase to levels seen in aluminium treated plants, thereby allowing malate build up. The increase in malate root tip concentration after 24 hours treatment did not occur in aluminium treated plants. This is probably due to the continued elevated malate exudation, which implies that the aluminium stress response of organic acids takes priority over the nutrient stress response in both *Brachiaria* species.

The two different exudate experiments show much higher malate concentrations in the *Brachiaria decumbens* exudate (70 μM) than *Brachiaria ruziziensis* exudate (5 μM in actual solution before adjusted for root mass). This is not due to higher exudation in *Brachiaria decumbens* than *Brachiaria ruziziensis,* but due to the sampling techniques employed. The lower *Brachiaria ruziziensis* measurements were taken from the bulk solution, where the malate has diffused into the surrounding solution, whereas the *Brachiaria decumbens* measurements represent actual malate concentration at the root tip surface. This root tip surface concentration is very high and would provide a formidable barrier to aluminium in solution around the root. An estimate for an average aluminium concentration in an acid soil is around 100 μM, similar to the levels used in these experiments.

5.5 Conclusions

Organic acid accumulation is not triggered by phosphate starvation although organic acids may play role in response of *Brachiaria decumbens* and *Brachiaria ruziziensis* to nutrient stress.

Brachiaria decumbens and *Brachiaria ruziziensis* employ two separate organic acid aluminium tolerance mechanisms. The first involves primarily a large induction of citrate in root tissue for internal detoxification and sequestering of aluminium. The second mechanism involves exudation of malate and oxalate for chelating and excluding aluminium from root tip cells.

The greater aluminium tolerance of *Brachiaria decumbens* and *Brachiaria ruziziensis* than *Tricium aestivum cv Atlas* is probably due to higher organic tissue concentrations, particularly citrate, and exudation of relatively high concentrations of oxalate.

Organic acid accumulation in root tip tissues does not explain the difference in aluminium tolerance between *Brachiaria ruziziensis* and *Brachiaria decumbens*.

Chapter Six: Oxidative Stress System

6.1 Introduction

6.1.1 Oxidative stress system

The antioxidant system is primarily involved in control of cellular levels of active oxygen species such as hydroxyl radicals, superoxide anions and hydrogen peroxide, all of which are extremely reactive and can damage all macromolecular components of cells such as DNA and hence are extremely toxic to cells (Pell *et al.,* 1997; Hagar *et al.,* 1996; Alscher *et al.,* 1997; Charles and Halliwell, 1981). Superoxide and hydrogen peroxide are produced by a number of activities including during photosynthesis, fungal and bacterial interactions and abiotic stresses such drought, ozone exposure, photodynamic herbicides (Mehlhorn *et al.,* 1990) or heavy metal stress, where Fenton type reactions involving transition metals such as iron or copper can take place leading to lipid peroxidation (reviewed by Low and Merida, 1996).

Hydrogen peroxide accumulation is involved as a signal molecule for triggering a general environmental and biotic stress response. It triggers gene expression and enzymes (Foyer *et al.,* 1997), including increasing activities of enzymes such as various peroxidases and activating secondary metabolism, leading to lignification and ultimately cell death (Alvarez and Lamb, 1997).

Superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) concentrations in the cell are controlled by a series of enzymes which convert superoxide to hydrogen peroxide and then hydrogen peroxide to water (Alscher *et al.,* 1997; Allen, 1995). Ascorbate and glutathione are used as proton acceptors providing reductant capacity (Foyer, 1993; Hausladen and Alscher, 1993; Hess, 1993; Winkler *et al.,* 1994). A further series of enzymes is then involved in recovery of ascorbate and glutathione from their reduced forms (Figure 6.1).

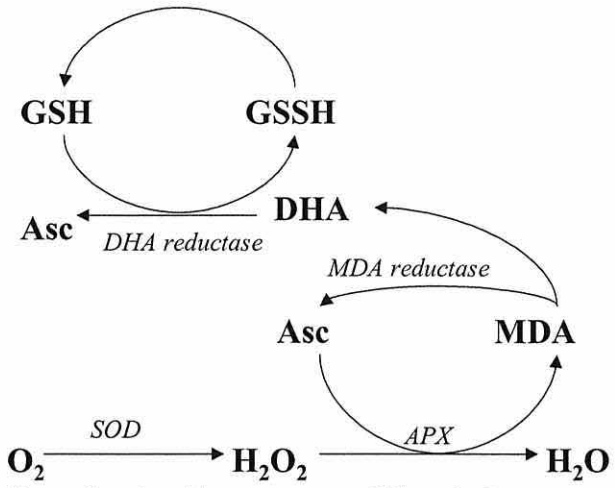

Figure 6.1 Outline of antioxidant system. Abbreviations:- superoxide (O_2^-), hydrogen peroxide (H_2O_2), water (H_2O), superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), ascorbate (Asc), monodehydroascorbate (MDA), monodehydroascorbate reductase (MDA reductase; EC 1.6.5.4), dehydroascorbate (DHA), dehydroascorbate reductase (DHA reductase; EC 1.8.5.1), glutathione (GSH) and reduced glutathione (GSSH). Based on Asada, 1994a.

Reduction of hydrogen peroxide to water can be catalysed by ascorbate peroxidase (as in Figure 6.1), catalase or general peroxidase enzymes (Allen, 1995). Ascorbate peroxidase is thought to be the primary enzyme responsible for this process in the antioxidant system (Asada, 1994b). Catalase has a much higher K_m value for its substrate than ascorbate peroxidase, which means that it is unable to decrease hydrogen peroxide levels to as low a concentration as attained by ascorbate peroxidase (Miyake *et al.*, 1991).

Ascorbate peroxidase is very similar in its amino acid sequence to cytochrome-c peroxidase found in cyanobacteria, but very different structurally to guaiacol peroxidases (Asada, 1992a). This may mirror a difference in their roles in cells. Ascorbate peroxidase is primarily involved in scavenging of hydrogen peroxide, whereas the numerous peroxidases which can use guaiacol as substrate are mainly involved in the biosynthesis of cell metabolites (Asada, 1994b), and other processes such as regulation of cell elongation (Asada 1994a).

The ascorbate used by ascorbate peroxidase is oxidised to monodehydroascorbate. This is then converted back to ascorbate by monodehydroascorbate reductase using either reduced ferridoxin (if taking place in the chloroplast) or NAD(P)H. Monodehydroascorbate (MDA) will spontaneously disproportionate if not converted quickly enough forming ascorbate and dehydroascorbate (DHA) (Allen, 1995; Asada, 1992b; Foyer and Mullineaux, 1994).

MDA + MDA → Ascorbate + DHA

Dehydroascorbate is scavenged by dehydroascorbate reductase to ascorbate using reduced glutathione as reductant (Allen, 1995; Asada, 1992b). Oxidised glutathione (GSSG) is then reduced by glutathione reductase using NAD(P)H (Allen, 1995; Asada, 1992b). Ascorbate and glutathione pool size can increase within minutes of onset of abiotic stress (Hausladen and Alscher, 1993; Foyer *et al.,* 1994).

6.1.2 Antioxidant system and aluminium tolerance

There is a possible role for the oxidative stress pathways in resistance to aluminium stress. Cakmak and Horst (1991) showed that aluminium treatment increased lipid peroxidation in soybean (*Glycine max*), and that there was a close relationship between lipid peroxidation and inhibition of root elongation. Lipid peroxidation was further increased by addition of iron. They also showed an increase in superoxide dismutase and peroxidase activity but a decrease in catalase activity with aluminium treatment.

Work by Richards *et al.* (1998) showed that several oxidative stress genes are induced by aluminium stress in *Arabidopsis thaliana,* including superoxide dismutase, peroxidase, glutathione-s-transferase. These enzymes are also triggered by treatment with ozone, an oxidative stress inducing pollutant, indicating that oxidative stress may play a role in plants reaction to aluminium.

Work in Japan on tobacco tissue cultures has further indicated a possible role of oxidative stress in response to aluminium treatment. Treatment with both aluminium and iron caused aluminium uptake, lipid peroxidation and loss of cell viability (Yamamoto *et al.,* 1997; Ono *et al.,* 1995). Accumulation of aluminium and loss of cell viability occurred simultaneously, followed by lipid peroxidation and iron uptake. However treatment with lipophillic antioxidants stopped lipid peroxidation, aluminium accumulation and loss of cell viability, indicating that lipid peroxidation may be responsible for loss of cell viability (Yamamoto *et al.,* 1997).

Phosphate starvation was also shown to reduce lipid peroxidation, accumulation of aluminium and iron and loss of cell viability (Yamamoto *et al.,* 1996). It is suggested that this was due to better protection of the plasma membrane, thereby preventing accumulation of aluminium. Phosphorus starvation was shown to increase the concentrations of beta carotene, an antioxidant carotenoid pigment.

Aluminium treatment causes a decrease in reduced glutathione and an increase in the oxidised form (Yamaguchi *et al.,* 1999). Reduction in glutathione synthesis by use of buthionine sulfoximine, which inhibits y-glutamyleysteine synthetase, caused higher lipid peroxidation, aluminium accumulation and loss of viability of tobacco cells under aluminium treatment. This suggests that glutathione is important in protecting cells from membrane damage caused by aluminium, either by its role as a reductant or through direct consumption/binding (Yamaguchi *et al.,* 1999). Copper uptake and assimilation in roots of *Phaseolus vulgaris* has been shown to cause lipid peroxidation and induction of catalase and ascorbate peroxidase activity in the leaves but had no effect on superoxide dismutase (Wecks and Clijsters, 1996).

Glutathione, in addition to its role in anti oxidation is also important as a building block in other biochemical processes, for example as a substrate for glutathione-S-transferase which enables the neutralisation of xenabiotics (Marrs, 1996) and also as a precursor for a family of phytochelating peptides which are involved in tolerance and sequestration of heavy metals such as cadmium. Zhu *et al.* (1999) have shown

that in Indian Mustard increasing activity of glutathione synthetase activity increased both the tolerance and accumulation of cadmium, a metal with very similar mechanisms of toxicity to aluminium. They found that the cadmium treated transgenic plant, which over-expressed the glutathione synthetase gene, had higher concentrations of glutathione, phytochelatins, calcium and thiol than cadmium treated wildtype plants.

If the oxidative stress system is involved in tolerance to aluminium, it would be expected that the reducing capacity would increase. Enzymes such as ascorbate peroxidase, guiacol peroxidases and superoxide dismutase would be expected to increase in activity. Metabolites providing reductant capacity such as ascorbate and glutathione would increase in concentration or a higher percentage would be occurring in the oxidised form.

As yet there is little experimental data on the oxidative stress response to aluminium. Much of the data thus far produced is circumstantial and most of it on cell culture rather than whole plant systems, but the data so far revealed indicate that this may be a potential aluminium tolerance mechanism.

6.1.3 Chapter aims

This chapter will examine whether the oxidative stress system in *Brachiaria decumbens* and *Brachiaria ruziziensis* is triggered by aluminium treatment as a potential defence mechanism to combat membrane peroxidation caused by aluminium toxicity. If the oxidative stress enzymes are involved as an aluminium tolerance mechanism then aluminium treatment should cause a significant increase in activity of key enzymes such as superoxide dismutase, peroxidases and ascorbate peroxidase.

6.2 Method

6.2.1 Pilot study

Brachiaria decumbens and *Brachiaria ruziziensis* seeds were surface sterilized using protocol used in previous experiments and germinated on filter paper in petri dishes in the dark at 25°C for 48 hours, then exposed to sunlight at 20°C for a further 24 hours before being transplanted to nutrient solution supported on 1mm mesh. The plants were grown in the usual hydroponic system described in Chapter Two, 6 seedlings per 600ml pot. The plants were then grown on for a further 30 days before the beginning of the treatment.

The 30-day old *B. Ruziziensis* and *B. decumbens* plants were treated with either 0 or 200 μM Aluminium. Each treatment had 7 replicate pots with 6 plants per pot, and the treatment was continued for 48 hours before harvesting the plants for experimentation. 20 ml of the nutrient solution from each pot was taken for pH testing. A sub-sample of five 8 mm root tips were taken from each pot and analysed for callose (see chapter 3 for method). The remaining roots were then washed using distilled water as previously described, separated from the leaves, placed in a labelled plastic bag and frozen in liquid nitrogen, then stored in a freezer at -50°C for 10 days. The frozen root samples were transported to Gottingen, Germany on dry ice for analysis of catalase, superoxide dismutase, and peroxidase enzyme activity. Samples remained frozen and stored at -50°C until extraction of enzymes.

6.2.2 Time course experiment

Fourteen-day old plants were treated with either 0 or 200 μM aluminium. Root tip (0-3cm from tip) samples were removed and extracted at 0, 6, 12, 24, 48 and 72 hours after initiation of treatment. Three replicates were taken for each treatment at each time point. Samples were extracted and analysed for catalase and guaiacol peroxidase activity and for ascorbate concentration.

6.2.3 Oxidative stress enzyme assays

6.2.3.1 Enzyme extraction

First optimum triton X-100 and polyvinylpolypyrrolidone (PVP) extraction concentrations were calculated by using extraction buffers with a range of PVP (0 - 400mg) and triton-X (0 - 2%) concentrations. Between 200 to 500 mg of frozen root tips (end 2 cm) from *B. decumbens* control treatment were ground up in a pestle and mortar using liquid nitrogen, and between 200 to 500 mg weighed out into centrifuge tubes containing 10 ml of 100 mM phosphate buffer solution pH 7.8 (K_2HPO_4 15.3 g/L, KH_2PO_4 1.496 g/L) containing either 0, 100, 200 or 400 mg PVP and 0, 0.5, 1, 1.5 or 2% triton-X. The samples were thoroughly mixed and stored on ice for at least 20 minutes to allow breakdown of membranes by triton-X and binding of phenols by PVP. The samples were then centrifuged at 20,000 rpm and 4°C for 60 minutes and the supernatant collected. The supernatant extract was then further cleaned using sephadex PD-10 gel columns (Pharmacia Biotech).

The columns were first eluted using 4 x 5 ml 50 mM phosphate buffer pH 7.8 (K_2HPO_4 7.65 g/L, KH_2PO_4 0.748 g/L), not allowing the level of the buffer to drop below the surface of the gel. 2.5 ml of supernatant extract was then added to columns and allowed to drain through. When the columns had stopped dripping the extract was eluted and collected using 3.5 ml 50 mM phosphate buffer pH 7.8. Extracts were then kept on ice or stored in the freezer at -30°C when not being used.

The SOD assay was then carried out to find the best extraction concentration of Triton-X and PVP. This was found to be 200 mg PVP and 1% triton-X. The treatment samples were then extracted as stated above. Extracts for peroxide activity (POD) assay were not filtered using the gel columns, the raw extract being clean enough to run the assay.

6.2.3.2 Superoxide dismutase (SOD) assay

Superoxide dismutase activity was measured using the photo-spectroscopic method described in McCord and Fridovitch (1969). A cuvette with the following was prepared:- 550 µl 50mM phosphate buffer pH 7.8 with 0.1 mM EDTA and 0.02 mM NaN_3, Xµl extract, 250 - Xµl 50mM phosphate buffer pH 7.8, 100 µl 0.5 mM xanthine (3.8025mg/50ml in 1M NaOH. Made fresh and stored on ice) and 50 µl 200 µM cytochrome-c (2.4 mg/ml in 50mM phosphate buffer pH 7.8. Made fresh and stored in dark on ice). For each sample three concentrations of extract were used; X = 150 µl, 200 µl and 250 µl, and for each extract concentration 3 replicates were measured. Three replicate blanks (50 mM phosphate buffer pH 7.8 instead of extract) were also measured per sample.

The reaction was started by addition of 50 µl xanthine oxidase (11 µl/ml in 50 mM phosphate buffer pH 7.8 with EDTA and NaN_3. Made fresh and stored on ice), and absorbance at 550 nm was measured every 15 seconds for 5 minutes at 25°C. For the pilot study in Gottingen, absorbance was measured using an Amersham pharmacia biotech Ultraspec 4000 uv/visible spectrophotometer running Swift II software for calculation of enzyme kinetics and at Bangor (Time course experiment) using a thermoSpectronic UV1 spectrophotometer with built in software.

Xanthine oxidase converts xanthine to uric acid releasing superoxide as by product, which reduces cytochrome-c. The assay measures the oxidation state of cytochrome-c. SOD in the sample convert superoxide to hydrogen peroxide thereby decreasing the rate of reduction of cytochrome-c. Rate of change in absorbance was averaged for the 3 replicates at each extract concentration and the average values were used to calculate SOD activity using the following equation:-

(A / mg of sample extracted x 1000) x 10 x 1.4 x 1000 / extract volume in cuvette

A = (absorbance of blank / absorbance of sample x 1000) - 1

NB. 10 is the volume of extraction buffer used to extract sample

1.4 is the volume of buffer used to elute the extract from the gel column divided by the volume of extract put through the column.

6.2.3.3 Guaiacol peroxidase (POD) assay

This is an assay for general peroxidase activity using a synthetic substrate, guaiacol, designed to act as substrate for a wide number of peroxidase enzymes (Putter, 1970). This does not include ascorbate peroxidase, which cannot use guaiacol as substrate.

General reaction:-

$$\textbf{guaiacol} + \mathbf{H_2O_2} \rightarrow \textbf{peroxidase} \rightarrow \mathbf{H_2O} + \textbf{guaiacol } \mathbf{H^+}$$

The following solutions were added to the cuvette 500 µl of 100 mM phosphate buffer pH 5.25 (K_2HPO_4 1.48 g/l, KH_2PO_4 12.45 g/l), 50 - X µl of 100 mM phosphate buffer pH 7.0 (K_2HPO_4 10.712 g/l, KH_2PO_4 5.24 g/l), X µl sample and 400 µl of 100 mM guaiacol (544µl 98% guaiacol solution, made up to 50 ml with distilled water). The reaction was started with the addition of 50 µl 200 mM H_2O_2 (1133 µL of 30% hydrogen peroxide solution, made up to 50 ml with distilled water) and the absorbance at 436 nm measured every 15 seconds for 5 minutes at 25°C. Extraction coefficient - 25.5 $mM^{-1}cm^{-1}$.

The assay measured the reduction of guaiacol, which turns brown. Each sample had 3 replicates, but no blank is needed. Hydrogen peroxide and guaiacol were made fresh, the hydrogen peroxide being kept on ice.

Calculation:-

(Average Absorbance x volume used to extract x 4 x total volume of solution in cuvette)

divided by

(Extinction coefficient x 60 x volume of extract in cuvette)

6.2.3.4 Catalase assay

Reaction equation:-

$$\mathbf{H_2O_2 \rightarrow catalase \rightarrow H_2O + H}$$

The method is as described in Aebi (1983). The following solutions were added to the cuvette. 600 µl of 50 mM phosphate buffer pH 7.0, 150-X µl of 50 mM phosphate buffer pH 7.8 and X µL Extract. The reaction was started upon addition of 250 µL of 40 mM H_2O_2 (226.5 µl of 30% H_2O_2 solution, diluted to 50 ml with 50 mM of phosphate buffer pH 7.0). Absorbance at 240nm was measured every 15 seconds for 3 minutes at 20°C, using crystal cuvettes.

Extinction coefficient 3.94 $M^{-1}cm^{-1}$.

The reaction was very quick therefore only 3 cuvettes were measured at once. The assay measured the decrease in the concentration of hydrogen peroxide. Catalase is inhibited by hydrogen peroxide, therefore samples with low catalase activity may needed the concentration of hydrogen peroxide to be reduced. Before running assay the concentration of the hydrogen peroxide solution was tested by running a blank. An absorbance reading between 0.550 and 0.520 was attained indicating correct concentration.

6.2.3.5 Ascorbate extraction and analysis

Root tips from both *Brachiaria decumbens* and *Brachiaria ruziziensis* were excised and immediately frozen in liquid nitrogen during the time course experiments and stored at -30°C until extraction. The root tips were then ground up with liquid nitrogen and approximately 80 mg (60 to 100 mg) of powdered tissue mixed with 1 ml of 2% (w/v) meta-phosphoric acid containing 1 mM EDTA and 1 mg PVP for every mg of sample. The sample was then centrifuged for 20 minutes at 30,000 g (4°C) and the supernatant used for analysis.

Analysis was by capillary zone electrophoresis (as described by Davey *et al.*, 1996) using a Beckman Coulter P/ACE MDQ capillary zone electrophoresis system with Gold 32 Karat software (Beckman Coulter). Capillary was fused silicon (Beckman Coulter) with 50 cm effective length (to detector window), and internal diameter 75 μm. Samples were injected hydrostatically for 5 seconds (0.5 psi). Separation was carried out at 25 kv for 11 minutes using a 100 mM Borate Buffer pH 9 (H_3BO_3, pH adjusted using 1M NaOH) as carrier electrolyte.

The ascorbate peak was identified by spiking with 250 μM ascorbate standard. The method only measured ascorbate in its reduced form. Total ascorbate root tip concentration was measured by reducing dehydroascorbate (DHA) to ascorbate then measuring as described above. Reduction of DHA was carried out by mixing 50 μL of sample with 75 μL of 60 mM dithiothreitol in 1M 2-[N-cyclohexylamino] ethansulphonic acid, then leaving for 60 minutes prior to analysis. DHA concentration was calculated by subtraction of reduced ascorbate from total ascorbate.

6.3 Results

6.3.1 Pilot Study

Treatment with aluminium caused an increase in callose content in root tips of both *Brachiaria decumbens* and *Brachiaria ruziziensis* (Figure 6.2). This indicates that the aluminium treatment used during the pilot study induced aluminium stress in both species.

Superoxide dismutase activity was species dependent, being significantly higher in *Brachiaria ruziziensis* than *Brachiaria decumbens* (p=0.007) (Figure 6.3). Superoxide dismutase activity was not affected by 48 hours of treatment with 200 μM aluminium in either *Brachiaria* species.

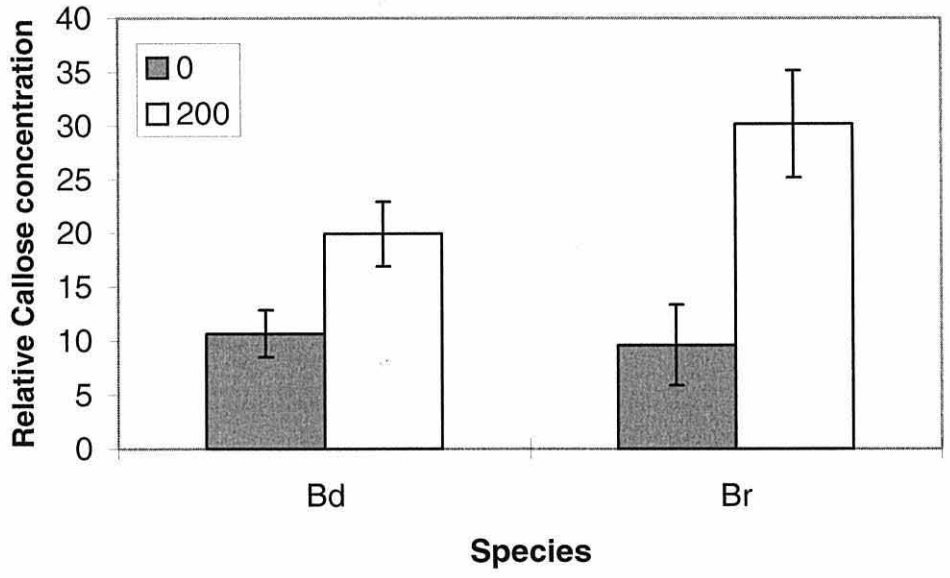

Figure 6.2: Relative callose content from root tips of *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br). Treated with 0 or 200 μM aluminium. ± S.E. n = 7.

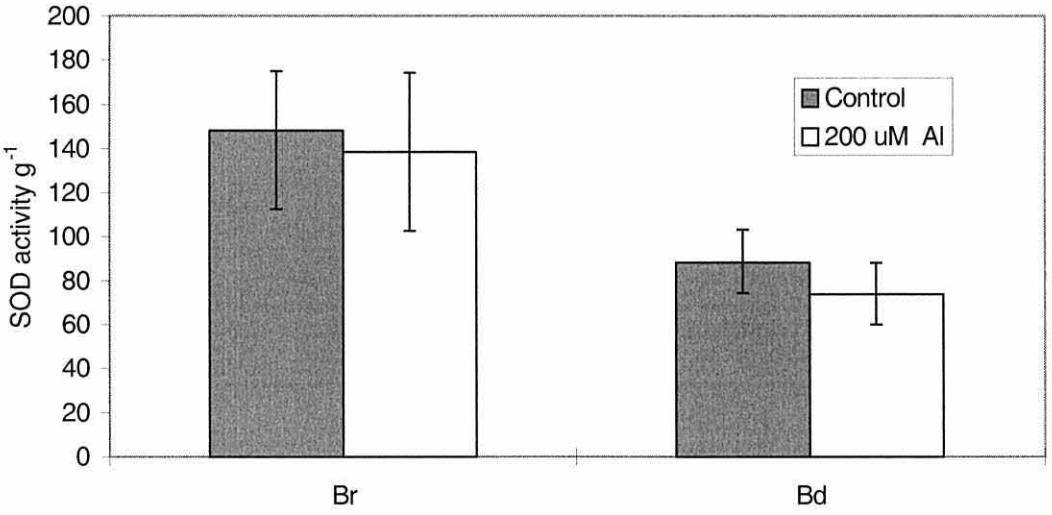

Figure 6.3: Superoxide dismutase activity (activity g^{-1}) in *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br), with 0 and 200 μM aluminium treatment. ± S.E. n = 7.

Peroxidase activity was higher in *Brachiaria decumbens* than *Brachiaria ruziziensis* under control conditions (p=0.135) (Figure 6.4). Peroxidase activity was decreased in *Brachiaria decumbens* after 48 hours of treatment with aluminium (p=0.078), to approximately 50% of the level in the control treatment. Peroxidase activity was also decreased, by approximately 30%, after 48 hours of treatment with aluminium in *Brachiaria ruziziensis*, but this decrease was not significant (p=297). The peroxidase activity after treatment with aluminium was approximately the same in both species.

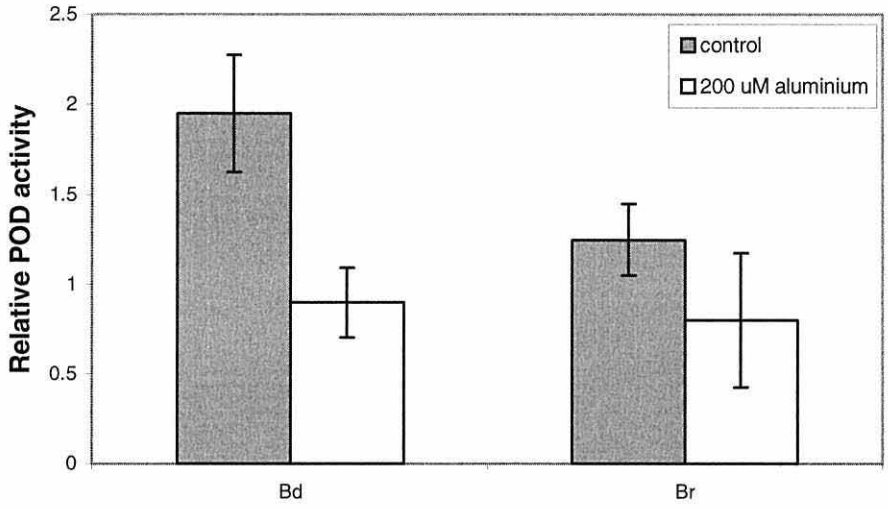

Figure 6.4: Guaiacol peroxidase activity (activity g^{-1}) in *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br). Treatment with 0 or 200 μM aluminium. ± S.E. n=7.

Catalase activity was extremely low and only just above the detection limit in both treatments and in both species. The data collected indicates no trends in catalase activity in respect to either species or aluminium treatment (Figure 6.5).

These results show that superoxide dismutase and peroxidase activity in these species is detectable by the technique used. Only 1 time point (48 hours), was used which does not give a clear indication of the processes occurring in the plants, however it does show that there may be a change in activity of the oxidative stress system in response to aluminium in *Brachiaria* species.

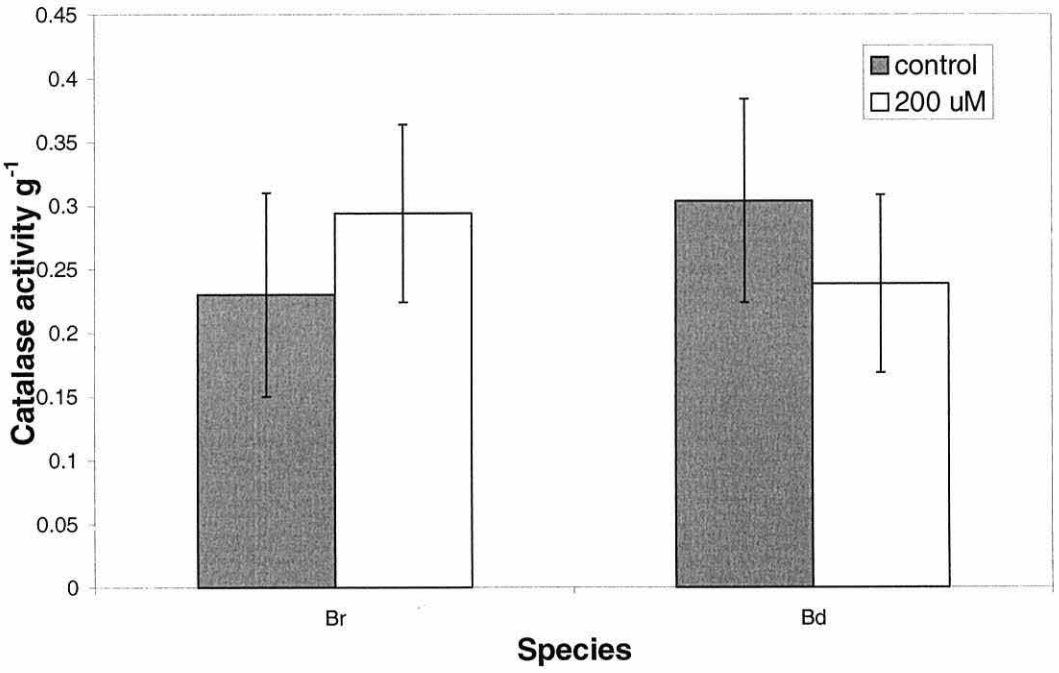

Figure 6.5: Catalase activity (activity g^{-1}) in *Brachiaria decumbens* (Bd) and *Brachiaria ruziziensis* (Br), treatment with 0 and 200 μM aluminium. ± S.E. n = 7.

6.3.2 Time course experiment

The time course data gave a much fuller picture than the single time point measurements made during the pilot study. The peroxidase data showed little response to aluminium treatment in either *Brachiaria* species (Figure 6.6), although there was a decrease in peroxidase activity in *Brachiaria ruziziensis* at the 24-hour time point ($p<0.001$), which recovered by the 48-hour measurement. As with the pilot study, the POD time course data showed that *Brachiaria decumbens* had significantly higher peroxidase activity than *Brachiaria ruziziensis* ($p=0.018$).

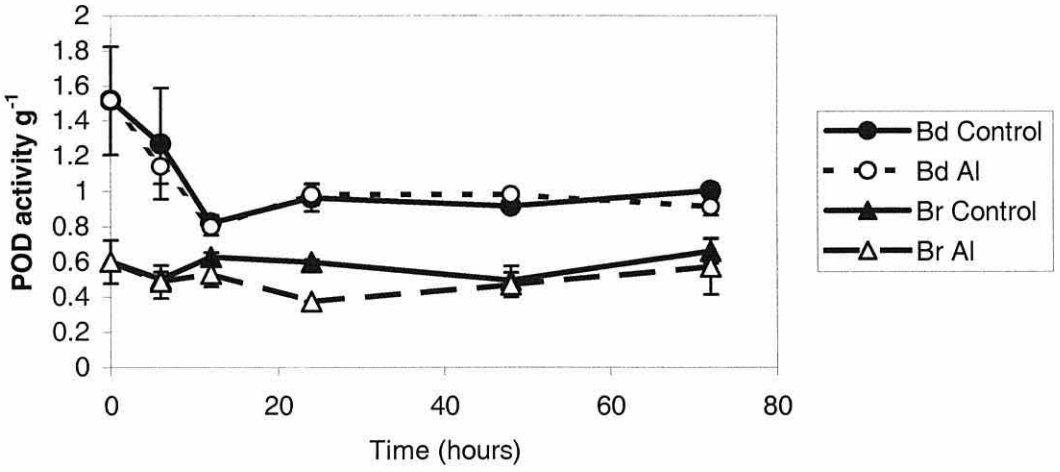

Figure 6.6: Guaiacol peroxidase activity (activity g^{-1}) over a 72 hour period in *Brachiaria decumbens* and *Brachiaria ruziziensis* treated with 0 or 200 μM aluminium. ± S.E. n = 3.

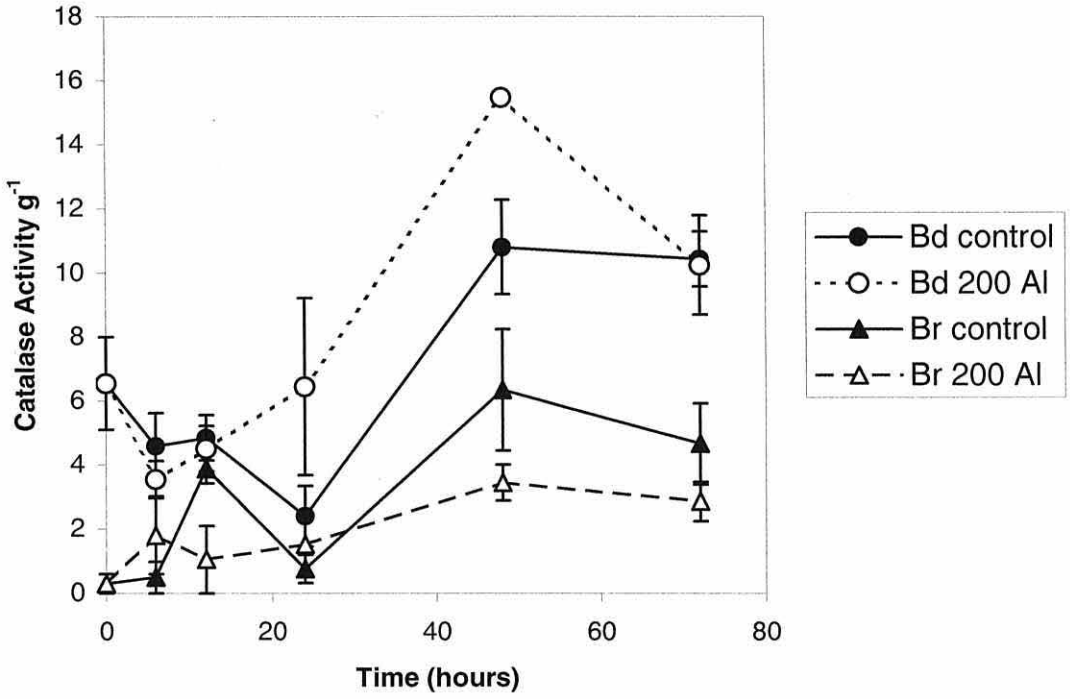

Figure 6.7: Catalase activity (activity g^{-1}) over a 72-hour period in *Brachiaria decumbens* and *Brachiaria ruziziensis* treated with 0 and 200 μM aluminium. ± S.E. n = 3.

Aluminium treatment caused an increase in catalase activity in *Brachiaria decumbens* after 24 hours ($p=0.036$), increasing by approximately 40% relative to control rates (Figure 6.7). This increase was maintained at 48 hours but declined to control levels at the 72-hour time point. Catalase activity in aluminium treated *Brachiaria ruziziensis* roots was generally the same or lower than activity in roots of control plants (Figure 6.7). Catalase activity was higher in *Brachiaria decumbens* than *Brachiaria ruziziensis* ($p<0.001$).

Aluminium had no effect on total ascorbate concentration in *Brachiaria ruziziensis* (Figure 6.8). In *Brachiaria decumbens* aluminium treatment did cause an increase in total ascorbate between 6 and 12 hours ($p<0.001$), however total ascorbate level had decreased back to control concentrations by the 24 hour measurement (Figure 6.8). There was no aluminium treatment effect on dehydroascorbate concentrations in either *Brachiaria* species (Figure 6.9).

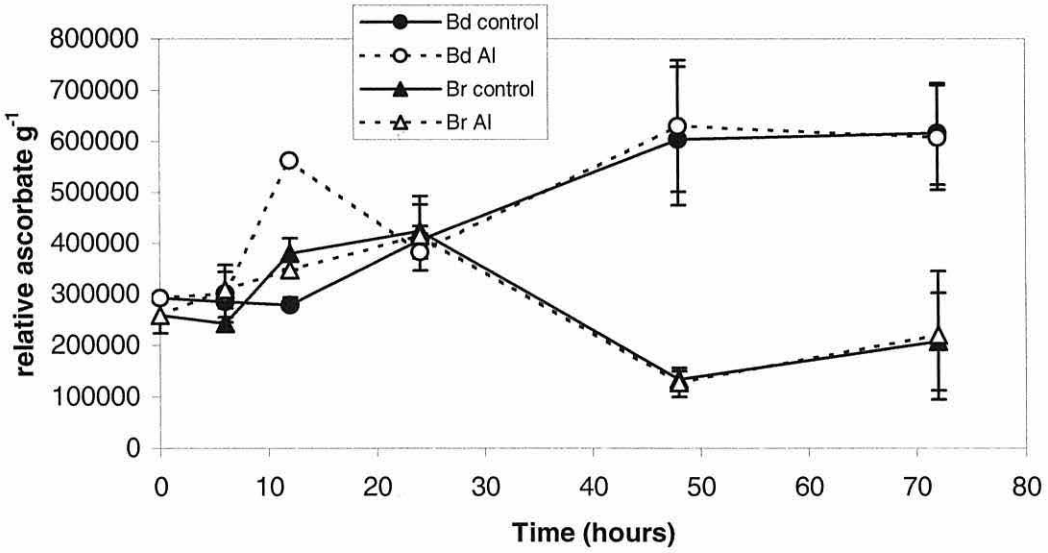

Figure 6.8: Effect of 0 or 200 μM aluminium on relative total ascorbate concentration over 72 hour period for *B. decumbens* and *B. ruziziensis*. ± S.E. n = 3.

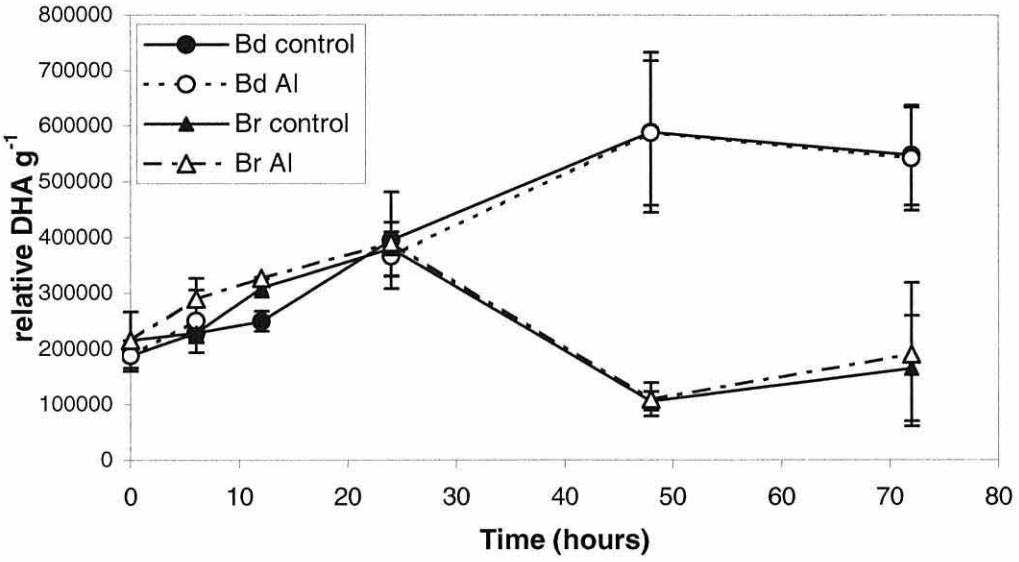

Figure 6.9: Effect of 0 or 200 μM aluminium on relative dehydroascorbate (DHA) concentration over 72 hour period for *B. decumbens* and *B. ruziziensis*. ± S.E. n = 3.

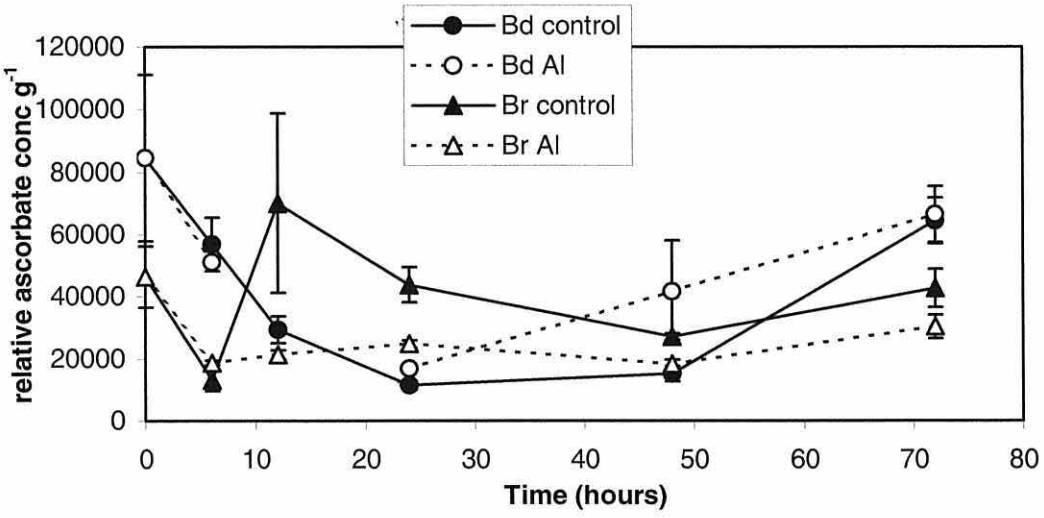

Figure 6.10: Effect of 0 or 200 μM aluminium on relative ascorbate concentration (reduced form) over 72 hour period for *B. decumbens* and *B. ruziziensis*. ± S.E. n=3.

There was high variability in reduced ascorbate concentrations over time in both species and under both 0 and 200μM aluminium treatment (Figure 6.10). Aluminium treatment causes a small increase in ascorbate levels in *Brachiaria decumbens* after 24 hours and further increased after 48 hours, then finally decreased to nearer control levels after 72 hours of aluminium treatment (Figure 6.10).

In *B. ruziziensis* aluminium caused a reduction in ascorbate concentration after 12 hours, to approximately half the control levels (Figure 6.10). The ascorbate levels then remained slightly lower in the aluminium treated *Brachiaria ruziziensis* plants than control plants (Figure 6.10)

6.4 Discussion

If the mechanism of aluminium toxicity is via lipid peroxidation and generation of active oxygen radicals within root cells, then for induction of the oxidative stress system to act as a potential aluminium tolerance mechanism there must be some visible change in the activities of key enzymes and concentrations of the reduced and oxidized forms of ascorbate and glutathione.

Aluminium has been shown to cause an increase in lipid peroxidation in a number of species including *Glycine max* (Cakmak and Horst, 1991) and *Nicotiana tabacum* (Yamamoto *et al.,* 1997; Ono *et al.,* 1995) particularly in the presence of iron. It has been suggested that aluminium increases lipid peroxidation by disrupting the membrane structure (Gutterage, 1997) causing the creation of active oxygen radicals, which may be due to the disruption of hemoproteins (Demopoulos, 1973).

In plants undergoing oxidative stress caused by aluminium treatment, an induction in activities of key enzymes has been seen, such as superoxide dismutase, peroxidases, ascorbate peroxidase and glutathione reductase (Cakmak and Horst 1991; Richards *et al.,* 1998). If aluminium is triggering an oxidative stress response in either *Brachiaria* species there should therefore be an increase in activities of enzymes

such as superoxide dismutase and consecutive enzymes such as peroxidases and ascorbate peroxidase. There should also be in initial increase in dehydroascorbate concentrations as ascorbate is converted to dehydroascorbate and monodehydroascorbate.

Treatment with 200 μM aluminium causes substantial inhibition of root elongation in *Brachiaria ruziziensis,* while not affecting root elongation in *Brachiaria decumbens.* For the oxidative stress system to act as a tolerance mechanism there should be a response seen in both species. A response in only *Brachiaria ruziziensis* would indicate that the oxidative stress system is triggered as a consequence of aluminium injury, as one of a number of processes initiated as aluminium overcomes the plants ability to cope, eventually leading to cell death.

The results show no increase in superoxide dismutase activity in either *Brachiaria decumbens* or *Brachiaria ruziziensis* in response to aluminium. This indicates there is no increase in superoxide anion production caused by aluminium treatment, which would be expected if aluminium was causing an increase in lipid peroxidation. Production of superoxide anions triggers an increase in superoxide dismutase activity (Elstner *et al.,* 1988).

There was no increase in activity of guaiacol peroxidases in response to aluminium treatment, which would be expected if the guaiacol peroxidases were involved in aluminium tolerance. In *Brachiaria ruziziensis* there was a significant decrease in peroxidase activity at the 24-hour time point, although activity recovered to control levels by the 48-hour measurement. Although peroxidases are not as important as ascorbate peroxidase in scavenging of hydrogen peroxide (Asada, 1994b), guaiacol peroxidase activities are usually increased several fold in response to oxidative stress caused by various stresses such as ozone, cadmium or aluminium (Cakmak and Horst, 1991; Richards *et al.,* 1998; Schutzendubel *et al.,* 2001).

Catalase activity, along with ascorbate peroxidase is triggered by accumulation of their substrate, hydrogen peroxide (Prasad *et al.,* 1994). Aluminium would therefore be expected to increase catalase activity. However, aluminium toxicity has been shown to cause a decrease in catalase activity in *Glycine max*, despite causing an increase in superoxide dismutase activity, and hence an increase in hydrogen peroxide concentration (Cakmak and Horst, 1991). This may indicate that aluminium has a direct inhibitory effect on catalase activity similar to the inhibitory effect caused by cadmium (Schutzendubel *et al.,* 2001). In both *Brachiaria* species catalase activity was very low and although an increase in activity was seen in *Brachiaria decumbens*, this increase was small relative to the increase caused by cadmium in *Pinus sylvestris* (Schutzendubel *et al.*, 2001), particularly considering the low level of catalase activity measured in *Brachiaria decumbens* and *Brachiaria ruziziensis*.

The activities of superoxide dismutase and peroxidase in both *Brachiaria* species is relatively low in comparison to the measurements made in *Glycine max* (Cakmak and Horst, 1991), although catalase activities were similar between *Glycine max* and the two *Brachiaria* species. This indicates that the *Brachiaria* species do not maintain oxidative stress enzyme activity at elevated or relatively high level.

In addition to the lack of response of key enzymes to 200 μM aluminium treatment, there was no significant aluminium effect on ascorbate or dehydroascorbate concentration. This all indicates that aluminium does not induce a positive response in the oxidative stress system in either *Brachiaria* species. This leads to the conclusion that the oxidative stress system does not act as an aluminium tolerance mechanism in *Brachiaria decumbens* or *Brachiaria ruziziensis.*

It also implies that 200 μM aluminium treatment does not cause a significant increase in lipid peroxidation. Higher aluminium treatment levels may well trigger a more substantial response in the oxidative stress system. Work by Cakmak and Horst (1991) found that lipid peroxidation was a consequence rather than the cause

of aluminium injury, which implies the requirement of sufficient aluminium to cause injury and lipid peroxidation prior to triggering the oxidative stress system.

6.5 Conclusions

The oxidative stress system does not act as an aluminium tolerance mechanism. 200 μM aluminium treatment is probably insufficient to cause a significant increase in lipid peroxidation.

Chapter Seven: Concluding Remarks

7.1 Differential response of Brachiaria decumbens, Brachiaria ruziziensis and Triticum aestivum to aluminium

Measurements of root growth in response to increasing aluminium treatment found *Brachiaria decumbens* to be unaffected by treatment with 200 μM aluminium, whereas in *Brachiaria ruziziensis* 200 μM aluminium treatment caused 40% root growth inhibition and in *Triticum aestivum* it caused 100% inhibition. This indicates that *Brachiaria decumbens* is significantly more aluminium tolerant than *Brachiaria ruziziensis*, which is in turn far more aluminium tolerant than *Triticum aestivum cv atlas.* This difference in species aluminium tolerance was supported by the measurement of callose induction in response to aluminium. However, callose induction was found to be a far more sensitive measure of aluminium toxicity than root growth inhibition, with increased concentrations of callose detected in *Brachiaria decumbens* treated with 200 μM aluminium, with no corresponding effect on root growth.

Examination of the effect of aluminium treatment on nutrient uptake in the two *Brachiaria* species found that the superior adaptation of *Brachiaria decumbens* to nutrient poor acid soil conditions compared to the closely related *Brachiaria ruziziensis* was probably due in part to a better nutrient uptake system, particularly iron, and lower optimum nutrient requirements. It is also probably also due in part to better maintenance of root and shoot nutrient concentrations when exposed to aluminium.

There was little increase in aluminium concentration in the shoot tissue of either *Brachiaria* species with increasing aluminium treatment, although aluminium concentrations do increase in the roots. This indicates that aluminium is not being transported to the shoots in *Brachiaria*, whereas increased aluminium concentrations are found in the shoots of *Triticum aestivum cv atlas*. This may imply either better

exclusion or internal detoxification mechanisms in the two *Brachiaria* species than in *Triticum aestivum cv atlas.* Internal aluminium concentrations do not account for the differential tolerance to aluminium of the two *Brachiaria* species

7.2 Aluminium tolerance mechanisms in Brachiaria decumbens and Brachiaria ruziziensis

Recent studies have implicated the oxidative stress system in aluminium tolerance (Cakmak and Horst, 1991; Yamamoto *et al.* 1997; Ono *et al.*1995; Richards *et al,* 1998). However aluminium was found to cause no increase in either superoxide dismutase, peroxidase or catalase activity in either *Brachiaria* species. Aluminium also had no significant effect on ascorbate and dehydroascorbate concentration, indicating that the oxidative stress system was not involved in aluminium tolerance in either *Brachiaria decumbens* or *Brachiaria ruziziensis.*

Organic acid accumulation and exudation has also been reported as a tolerance mechanism in response to aluminium in a wide variety of species (e.g. Ryan *et al.*, 1993; Hue *et al.,* 1986; Ma *et al.,* 1997c) and also been implicated as a mechanism to aid uptake of some nutrients (particularly iron and phosphate) in some species (e.g. Gardiner *et al.*, 1983 and Lopez-Millan *et al.*, 2001).

Organic acids were accumulated in root tip tissue in response to general nutrient deficiency in *Brachiaria decumbens* and *Brachiaria ruziziensis.* Organic acid accumulation was not triggered by phosphate starvation in *Brachiaria decumbens* or *Brachiaria ruziziensis*, although there was a synergistic increase in malate and citrate induction in the root tip tissue in response to a combination of aluminium treatment and phosphate starvation.

Brachiaria decumbens and *Brachiaria ruziziensis* were found to use organic acids as a defence against aluminium in two separate organic acid aluminium tolerance mechanisms. The first primarily involved a large induction of citrate in root tissue

for internal detoxification and sequestering of aluminium. The second mechanism involved exudation of malate and oxalate for chelating and excluding aluminium from root tip cells.

The specific exudation of oxalate in preference to citrate, which was at much higher concentrations in the tissue, indicates close control of exudation rather than passive diffusion. There may also be a high degree of spatial compartmentalisation of different organic acids within the cell.

The greater aluminium tolerance of *Brachiaria decumbens* and *Brachiaria ruziziensis* than *Triticum aestivum cv Atlas* was probably be due to higher organic tissue concentrations, particularly citrate, enabling higher internal aluminium tolerance. It may also be partly explained by exudation of relatively high concentrations of oxalate and malate. Organic acid accumulation in root tip tissues did not account for the difference in aluminium tolerance between *Brachiaria ruziziensis* and *Brachiaria decumbens*. EDTA studies found there to be significant accumulation of aluminium in the symplast compared to apoplast in *Brachiaria.* Symplastic aluminium was found to be primarily bound to citrate, although due to the high internal malate concentrations a significant proportion was bound to malate.

Looking at the plant responses to aluminium over time, it was found that callose induction occurred prior to inhibition of root growth (2 - 4 hours compared to 4 - 6 hours) in both *Brachiaria* species. Induction of increased organic acid tissue concentrations occurred faster in *Brachiaria ruziziensis* (4 to 10 hours) than in *Brachiaria decumbens* (10 to 24 hours). The increase in organic acids in the root tissue in both *Brachiaria* species appears slow in relation to both callose induction and root growth inhibition. However a fast induction of internal organic acid concentration is probably not important because there are high concentrations of some organic acids (e.g. malate) in the root tips in control conditions.

Organic acid tissue measurements, due to their mainly vacuolar nature, do not necessarily correspond with exudation fluxes. The exudation response may therefore be far more rapid than the induced increase in internal organic acid concentrations. The *Brachiaria ruziziensis* exudation data shows an induction of both malate and oxalate within 6 hours of treatment with aluminium. However, the exudation response is probably far quicker than six hours, as the measurement technique detects the organic acids only when they have reached a high enough concentration in the surrounding bulk solution.

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