Investigations into Aluminium Toxicity and Resistance in *Cynodon dactylon* using *In Vitro* Techniques

by

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ABSTRACT

Aluminium toxicity is a significant limiting factor to agricultural crop production globally, promoting the need for plants that are resistant to low pH and high Al^{3+}. Current literature suggests that Al^{3+} inhibits plant growth by stopping root elongation. Although considerable research has been directed towards Al^{3+}-inhibited root growth, the initial cellular targets and primary mechanisms of Al^{3+} toxicity still remain unclear. The present study, therefore, considered an alternate approach to investigating Al^{3+} toxicity and Al^{3+} resistance. Callus, a group of actively dividing meristematic cells, was exposed to Al^{3+} and the influence of Al^{3+} on callus growth was investigated.

In South Africa, gold mining results in the production of wastes that require vegetation cover resistant to low pH and high Al^{3+}, in order to promote stabilisation and prevent erosion. *Cynodon dactylon* was considered a key species for such a purpose since small populations of this grass were found growing on the acidic gold tailings. Different *C. dactylon* genotypes were exposed to Al^{3+} and the feasibility of using differences in callus growth to identify potential Al^{3+}-resistant individuals was assessed. An *in vitro* method for indirect somatic embryogenesis was concurrently established to regenerate whole plants from such calli.

Embryogenic calli were initiated from young leaf segments, using 2,4-D. Somatic embryo maturation and plant regeneration were achieved on a hormone-free Murashige and Skoog (MS) nutrient medium. In addition to this protocol for micropropagation via indirect somatic embryogenesis, nodal cuttings, on a single hormone-free MS nutrient medium, were shown to be suitable explants for micropropagation via direct organogenesis, albeit resulting in low plantlet yields (1 plant/explant).

In the investigation of Al^{3+} resistance, each of the three parameters tested (genotype, Al^{3+} concentration and exposure time) had a significant influence on callus growth rate. The nutrient medium supporting callus growth was modified in order to ensure known concentrations of free Al^{3+} ions (0.08-2.3 mM). This was achieved through the use of a

chemical speciation model (MINTEQA2). Fresh callus mass measurements for three genotypes were recorded at two-weekly intervals for a total of 8 weeks. Significant differences in callus growth rate were used to identify the genotypes as Al^{3+}-sensitive (Al-S), moderately Al^{3+}-resistant and Al^{3+}-resistant (Al-R), suggesting that it is feasible to use undifferentiated meristematic callus cells to screen for resistance to Al^{3+}.

In addition to callus growth rate, it was also possible to differentiate between the Al-S and the Al-R genotype using differences in cell numbers. Exposure to 0.8 mM Al^{3+} for 2 weeks resulted in an 88% reduction in the Al-S meristematic cell number whereas no Al^{3+} concentration tested had a significant inhibitory effect on the Al-R cell number. Aluminium was detected inside the callus cells, with the Al-S cells accumulating three times more Al in the nucleus than did the Al-R cells. It is suggested, therefore, that Al^{3+} inhibited meristematic cell number in the Al-S genotype by interfering with cell division. Two possible mechanisms by which the Al-R genotype was able to exclude Al^{3+} from its cells were investigated. The Al-R callus was able to maintain a higher extracellular pH (4.34 in Al-R and 4.08 in Al-S) and immobilise more Al in the cell wall (33% more in the Al-R) than the Al-S genotype.

The present study has developed a valuable tool for investigating the physiological effects of Al^{3+} on actively dividing meristematic cells. In addition, the somatic embryogenesis route allows for the concurrent *in vitro* selection and plantlet regeneration of genotypes of interest. Future work is necessary to confirm that the properties of undifferentiated cells in culture are maintained by the *ex vitro* whole mature plants.

PREFACE

The experimental work described in this thesis was conducted in the School of Life and Environmental Sciences, University of KwaZulu-Natal, Howard College Campus, Durban, under the supervision of Professors J.A Cooke and M.P Watt.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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List of Abbreviations

Al	aluminium
Al^{3+}	free aqueous Al ion
Al-R	aluminium-resistant
Al-S	aluminium-sensitive
Al-T	aluminium-tolerant
ABA	abscisic acid
BA	benzyladeninepurine
°C	degrees celsius
cm	centimetre
cm^3	cubic centimetre
cmol. l^{-1}	centimolar per litre
CO_2	carbondioxide
CS	citrate synthase
d	day
2,4-D	2,4-dichlorophenoxyacetic acid
DTZ	distal transition zone of the root apex
EDTA	ethylenediaminetetra-acetic acid
EDXM	energy dispersive X-ray microanalysis
fm	fresh mass
g	gram
g g^{-1}	gram per gram
g kg^{-1}	gram per kilogram
g l^{-1}	gram per litre
h	hour
IAA	indole acetic acid
IBA	indole butyric acid
kinetin	6-furfurylaminopurine
keV	kilo electron volt
kV	kilovolt

m	month
M	molar (mole. dm^{-3})
MFs	microfilaments
mg kg^{-1}	milligram per kilogram
mg l^{-1}	milligram per litre
min	minute
ml	millilitre
ml l^{-1}	millilitre per litre
mm	millimetre
mM	millimolar (mmole. dm^{-3})
mM g^{-1}	millimolar per gram
MS	Murashige and Skoog (1962) nutrient formulation
MTs	microtubules
nm	nanometre
nM	nanomolar (nmole. dm^{-3})
NO_X	nitrogen oxides
Pa	Pascal
PEG	polyethleneglycol
pM	picomolar (pmole. dm^{-3})
ppm	parts per million
rpm	revolutions per minute
s	second
SO_2	sulphurdioxide
µg g^{-1}	microgram per gram
µM	micromolar (mmole. dm^{-3})
μm^2	micrometre squared
µmol. m^{-2} s^{-1}	micromole per metre squared per second
v/v	volume per volume
w	week
wt %	weight percentage
y	year

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

One of the major constraints to global crop production is soil acidity (Eswaran *et al.*, 1997; Duncan, 2000; Sumner, 2001). Low productivity of acid soils usually results from aluminium toxicity that impacts negatively on root growth and function. Aluminium solubility is increased in acid soils that are below a pH value of about 5.5 (von Uexküll and Mutert, 1995; Clark and Baligar, 2000; Sumner, 2001). In many developed countries it is common agronomic practice to apply lime to reduce soil acidity and Al toxicity. However this approach can be expensive and is not considered an effective strategy for alleviating subsoil acidity (Kochian, 2000; Sumner, 2001). The adaptive response of plants to Al is a major factor in the widespread distribution of wild plants on acid soils. Natural forests and grasslands cover the largest areas of acid soils, representing important genetic resources with natural populations of plants resistant to Al and low pH (von Uexküll and Mutert, 1995). The ability of plants to grow in areas with high Al and low pH is the current focus of research by many groups around the world, since an improved understanding of Al toxicity and resistance will enable the selection of Al resistant crops and other useful plant species (Sumner, 2001; Duncan, 2000). This chapter reviews the chemistry of aluminium, the influence of Al toxicity on plant growth and the responses of Al resistant plants. It also introduces the chosen plant material and alternate means for plant propagation and outlines the aims and objectives of the present study.

1.2 Aluminium in soils

Aluminium is the most abundant metal and the third most common element of the earth's crust and constitutes approximately 8% (by weight) of the earth's crust (Driscoll and Schecher, 1990). The richest source of Al is aluminium trihydrate or bauxite. Aluminium also combines with silicon to form aluminosilicates, which are the major constituent of many rocks, clays and other soil minerals (Barnhisel and Bertsch, 1982). The total global surface area of acid soils are estimated to be between 3.77×10^7 km^2 (Eswaran *et al.*, 1997) and 3.95×10^7 km^2 (von Uexküll and Mutert, 1995) and this represents about 30% of the total ice-free land area. The major areas of Al toxic topsoils occur in South America, Africa

and North America (von Uexküll and Mutert, 1995; Eswaran *et al.*, 1997). World acid soils have been classified into eight groups: entisols; inceptisols; andisols; spodosols; alfisols; ultisols; oxisols; and histosols (von Uexküll and Mutert, 1995). Forty % of the total acid soils are oxisols and ultisols, characterised by oxides of Fe and Al.

Under natural conditions, soil acidity develops mainly as a result of weathering of alumino-silicate minerals by carbonic acid (Clark and Baligar, 2000). The weathering of parent material is driven by the continuous production of CO_2 in soil, by microbial root respiration and the leaching of soluble products. Parent rocks weather to form acid soils and the rate of acidification depends mainly upon the nature of the parent material, rainfall and temperature. Highly siliceous materials weather more slowly than more basic substrates, with both increased rainfall and temperature promoting this process (Sumner, 2001). Acid soils are generally categorised into four groups: 1) slightly acidic soils (pH 5.5 – 6.5), 2) moderately acidic soils (pH 4.5 - 5.5), 3) highly acidic soils (pH 3.5 – 4.5) and 4) extremely acidic soils (pH < 3.5) (Clark and Baligar, 2000). Soils in the first group do not limit plant growth since in that pH range there is an adequate supply of essential nutrients. However, those soils are prone to further acidification through poor management (i.e. high use of N fertilisers) and environmental pollution (SO_2, NO_x). In the second group of soils the availability of Ca, Mg, K and P is reduced and that of Al^{n+} increased, resulting in the formation of various pH dependent Al species. The same is true for the remaining groups (three and four), with these soils having high available Al^{n+} and low availability of essential nutrients. As soil pH decreases (below 5.5) increasing proportions of cationic exchange sites on clay minerals become occupied with Al^{n+}, by replacing other cations such as Mg^{2+}, Ca^{2+} and K^{+}. Increasing Al saturation of soil cation exchange sites are associated with decreasing pH and poor soil nutrient status (Clark and Baligar, 2000; Sumner, 2001).

1.3 Al in solution

Aluminium in soil solution is largely derived from cation-binding sites, dissolution from amorphous mineral phases and decomposition of organic matter (Driscoll and Schecher, 1990). Although Al in solution represents a small fraction of the total Al in the soil, it is the most important chemically and biologically available form (Driscoll and Schecher, 1990).

The free aqueous Al ion is associated with six water molecules in an octahedral configuration that is represented by the formula $Al(H_2O)_6^{3+}$ (Nordstrom and May, 1989). This form is conventionally referred to as Al^{3+}. The high positive charge of the trivalent Al ion induces a tight primary hydration shell. The hydrolysis of Al^{3+} involves the progressive loss of the hydration shell protons to water molecules in solution (Bertsch and Parker, 1996). The four hydrolysis reactions and the predicted availability of the various Al species at different pH values are shown in Table 1.1. In acidic solutions (< pH 4) Al^{3+} dominates but as the pH increases Al^{3+} undergoes successive deprotonations to form $Al(OH)^{2+}$ and $Al(OH)_2^+$ (Table 1.1). Solutions with a neutral pH generate a gibbsite precipitate ($Al(OH)_3$), that redissolves in basic solutions to form aluminate ($Al(OH)_4^-$). In the aqueous phase Al^{3+} may also form complexes with a variety of inorganic (OH^-, F^-, SO_4^-, PO_3^{3-}, H_4SiO_4; HCO_3^-) or organic ligands. The extent of such complexes depends upon the availability of Al^{3+}, solution pH, concentrations of ligands, ionic strength, and temperature (Driscoll and Schecher, 1990).

Table 1.1 Mononuclear hydrolytic aluminium species

Reaction		Species formed	pH
Al^{3+}			< 4
$Al^{3+} + H_2O$	=	$AlOH^{2+} + H^+$	4-5
$Al^{3+} + 3H_2O$	=	$Al(OH)_3^0 + 3H^+$	4-7
$Al^{3+} + 2H_2O$	=	$Al(OH)_2^+ + 2H^+$	5.5-7
$Al^{3+} + 4H_2O$	=	$Al(OH)_4^- + 4H^+$	> 7

Waters of hydration are omitted for simplicity. Hydrolysis reactions were taken from Nordstrom and May (1989) and pH values from Driscoll and Schecher (1990).

Polynuclear Al is defined as any species, complex or an aggregation of mononuclear Al (i.e. solid-phase $Al(OH_3)$) that contains more than one Al atom (Kinraide, 1991). Examples include: $Al_2(OH)_2^{4+}$; $Al_3(OH)_8^+$; $Al_6(OH)_{12}(H_2O)_{12}^{6+}$; and $Al_{13}O_4(OH)_{24}(H_2O)_{12}^{7+}$ (Bertsch and Parker, 1996). The most important polynuclear species with regard to phytotoxicity is triskaidekaaluminium ($Al_{13}O_4(OH)_{24}(H_2O)_{12}^{7+}$), commonly referred to as Al_{13} (Bertsch,

1989; Kinraide, 1991). Polynuclear species can be formed when the total Al activity increases or if the pH level increases in acidic solutions or decreases in basic solutions (Bertsch, 1989; Parker *et al.*, 1989a).

1.4 Phytotoxic Al form

The issue of identifying rhizotoxic chemical forms of Al has been a contentious one because, as discussed above, Al species change with fluctuating pH and form complexes with inorganic and organic soil ligands. The transition from a rhizosphere with low pH and high Al^{3+} to the intracellular pH with the cytomatrix having a neutral pH clearly represent a gradient of change which makes the speciation of Al within the cell problematic. The majority of the investigations on Al speciation and phytotoxicity were done using plant species of agronomic importance, in particular wheat (Kinraide and Parker, 1987ab, 1989; Kinraide, 1990, 1991, 1997). From that work, toxicity has been confirmed for only one polynuclear form, Al_{13}, and one mononuclear form, Al^{3+} (Parker *et al.*, 1989b; Kinraide, 1997). Wheat cultivars exhibited differential tolerance to Al^{3+} but not to Al_{13}, suggesting a fundamental physiological difference in the toxicity of the two types of Al. Mononuclear Al-hydroxy species (i.e. $Al(OH)^{2+}$ and $Al(OH)_2^{+}$) failed to reveal any toxicity for wheat but have been reported to inhibit root growth in four dicotyledonous species (Kinraide and Parker, 1990). In an earlier study aluminate ions ($Al(OH)_4^-$) were found to be non-toxic to wheat (Kinraide, 1990), however, this has been recently disputed by Ma *et al.* (2003) who showed that $Al(OH)_4^-$ is toxic to wheat in an alkaline growth solution (pH > 9).

Although Al_{13} was reported as the most phytotoxic Al form for wheat roots (Kinraide, 1997), most studies have focused on Al^{3+} since Al_{13} was considered to be unstable and precipitates out of solution easily to form gibbsite ($Al(OH)_3$) (Bertsch and Parker, 1996). Further, soil clays and organic matter are thought to have a strong affinity for polynuclear Al species and it is generally difficult for these species to exchange into solution. It is therefore believed that the Al^{3+} status in the soil solution is the best predictor of phytotoxicity (Kochian, 1995; Matsumoto *et al.*, 2001). The focus of the present study was therefore on Al^{3+}.

1.5 Al Toxicity

The inhibition of root growth, caused mainly by interactions of Al within the root apex, has been recognized as the most important component of Al phytotoxicity (Kochian, 2000; Čiamporová, 2002). For this reason root growth analysis has been used in numerous studies to screen for resistance to Al in plant species. Aluminium-injured roots are characteristically thickened, stubby, brown, brittle and occasionally necrotic. Inhibition in the elongation of the main root axis, initiation of lateral roots close to the apex of the main axis and no lateral root growth, give roots of Al-stressed plants a corralloid appearance (Clarkson, 1969; Andersson, 1988; Taylor, 1988). The root tip appears to be the primary target for Al^{3+} toxicity and application of Al to any other region of the root has no effect on root growth (Delhaize *et al.*, 1993a; Ryan *et al.*, 1993).

Severe Al toxicity damages the root system, making plants susceptible to drought stress and mineral nutrient deficiencies. In some plant species toxicity symptoms are similar to that of phosphate deficiency (small dark green leaves, late maturation, purple/red colouration of stems and leaves, chlorosis and necrosis of leaf tips), and in other species symptoms include petiole collapse, mottled chlorosis and necrosis (Foy *et al.*, 1978; Andersson, 1988). Bennet and Stewart (1999) in ryegrass (*Lolium perenne*) showed that although Al was not transported to the shoot it appeared to affect plant water relations. The response of ryegrass to Al was linked to the regulation of its internal water transport, with the Al-S (Al-sensitive) genotype unable to control the Al-stimulated water flux. Other shoot-related effects of Al include reductions in growth and nutrient element concentrations (Fageira and Santos, 1998; Purcino *et al.*, 2003) and chlorophyll content and photosynthetic rate (Paliwal and Sivaguru, 1994; Lidon *et al.*, 1997; Milivojević and Stojanović, 2003).

At the cellular level, possible targets for Al^{3+} include the cell wall (apoplast), plasma membrane or components in the cytoplasm (symplast) (Kochian, 1995; Matsumoto, 2000; Čiamporová, 2002). A number of critical cellular functions are involved during cell elongation and division and Al probably interferes with many of these functions thereby inhibiting root growth. Table 1.2 summarises twenty-one of the many published studies to

Table 1.2 Proposed mechanisms of Al toxicity

Species	Plant material	Al target	Al-induced effect	Cellular response	Whole plant response	Reference
Triticum aestivum	root	nucleus	Al binds to DNA and inhibits synthesis	cell division inhibited	root elongation inhibited	Wallace and Anderson, 1984
Zea mays	root	nucleus	Al induces extrusion of nucleolar material	cell division inhibited	root elongation inhibited	Liu and Jiang, 2001
Z. mays	cell suspension	cell wall	Al binds and crosslinks pectin matrix in cell wall	permeability of cell wall reduced, CD inhibited	-	Schmohl and Horst, 2000
Z. mays	cell suspension	cell wall	Al binds pectin, cw permeability reduced	exudation and activity of acid phosphatase reduced		Schmohl and Horst, 2002
	root	cell wall	Al binds pectin, cw permeability reduced	exudation and activity of acid phosphatase reduced	root elongation inhibited	
T. aestivum	root	cell wall	Al induces cell wall polysaccharides	thickens and rigidifies cell wall	root elongation inhibited	Tabuchi and Matsumoto, 2001
Hordeum vulgare	root	cell wall	Al inhibits cellulose synthesis	cells expand laterally not longitudinally	root elongation inhibited	Teraoka *et al.*, 2002
N. tabacum	cell suspension	plasma membrane	Al blocks Ca^{2+} channel in plasma membrane	interferes with Ca^{2+} homeostasis, CD inhibited	-	Jones *et al.*, 1998

Table 1.2 contd.

Species	Plant material	Al target	Al-induced effect	Cellular response	Whole plant response	Reference
Curcurbita pepo	root	plasma membrane	Al-induced depolarization of PM	H^+-ATPase activity and H^+ efflux inhibited	root growth inhibited	Ahn *et al.*, 2001, 2002
Nicotiana tabacum	cell suspension	plasma membrane	Al enhances Fe(II) mediated lipid peroxidation	loss of membrane integrity, leads to Al acccumulation and cell death	-	Ono *et al.*, 1995; Ikegawa *et al.*, 1998; Chang *et al.*, 1999
Pisum sativum	root	plasma membrane	Al-induced lipid peroxidation	loss of plasma membrane integrity	root elongation inhibited	Yamamoto *et al.*, 2001
T. aestivum	root	plasmodesmata	Al-induced callose deposited at PD	blocked PD interferes with cell-to-cell transport	root elongation inhibited	Sivaguru *et al.*, 2000
N. tabacum	cell suspension	mitochondria	mitochondrial activity reduced	reactive O_2 species produced, ATP reduced	-	Yamamoto *et al.*, 2002
Pisum sativum	root	mitochondria	mitochondrial activity reduced	reactive O_2 species produced, ATP reduced	root growth inhibited	Yamamoto *et al.*, 2002
Glycine max	cell suspension	actin network	increased rigidity of actin network	cell division inhibited	-	Grabski and Schindler, 1995

Table 1.2 contd.

Species	Plant material	Al target	Al-induced effect	Cellular response	Whole plant response	Reference
T. aestivum	root	microtubules	disruption of MT network	longitudinal cell expansion inhibited	root elongation inhibited	Sasaki *et al.*, 1997
Z. mays	root	microtubules	MTs reorganized in inner cortex cells, MTs stabilised in outer cortex cells	cell expansion inhibited	root elongation inhibited	Blancaflor *et al.*, 1998
Z. mays	root	plasma membrane microtubules	Al-induced depolarization of PM, depolymerization of MTs	cell expansion inhibited	root elongation inhibited	Sivaguru *et al.*, 1999a
N. tabacum	cell suspension	cortical microtubules	CMTs depolymerised in log phase cells and stabilised in stationary phase cells	structural integrity lost in log phase cells, cell division inhibited	-	Sivaguru *et al.*, 1999b
N. tabacum	cell suspension	cortical microtubules	Al-induced disruption of CMTs	decreased cell viability	-	Schwarzerova *et al.*, 2002

CD = cell division, CW = cell wall, PM = plasma membrane, PD = plasmodesmata, ATP = adenosine triphosphate, MT(s) = microtubule(s), CMTS = cortical microtubules

illustrate the relationship between the cellular target of Al, the biochemical effects, and the cellular responses.

A number of the early studies linked Al inhibited root growth with a reduction in cell division (Clarkson, 1965; Matsumoto *et al.*, 1976; Horst *et al.*, 1983). The negative effects of Al on cell division were first demonstrated using onion (*Allium cepa*) roots (Clarkson, 1965). It was shown that a disappearance in mitotic figures was closely related to Al induced cessation of root growth and it was suggested that some mechanism associated with cell division was highly sensitive to Al^{3+}. Later studies showed that Al^{3+} was accumulated in the nuclei of root cells, formed a preferential complex with DNA and inhibited DNA synthesis (Matsumoto *et al.*, 1976; Wallace and Anderson, 1984). Aluminium also appeared to affect the integrity of the nucleolus causing the extrusion of nucleolar material into the cytoplasm (Fiskesjö, 1983; Liu and Jiang, 1991). Recent studies have shown that high concentrations of Al (0.1 M) induced aberrations in the chromosome morphology of *Zea mays* root tip cells which resulted in reduced mitosis (Liu and Jiang, 2001).

Although a number of reports confirmed the link between Al inhibited root growth and reduced mitotic activity, the primary mechanism of Al toxicity is still not clear (Kochian, 1995; Čiamporová, 2002; Samac and Tesfaye, 2003). Aluminium located in the nuclei of meristematic root tip cells has been shown to decrease mitotic activity (Silva *et al.*, 2000; Liu and Jiang, 2001). This decrease in mitotic activity usually occurred several hours after exposure to Al, for example after 6-8 h in *Allium cepa* (Clarkson, 1965) and 24 h in *Zea mays* (Liu and Jiang, 2001). However, root growth inhibition can be shown to occur in a much shorter time, for example 1 h after exposure in *Glycine max* (Kataoka and Nakanishi, 2001). As the cell cycle usually takes several hours to be completed, it appears that the primary target for Al^{3+} is cell elongation. This is compatible with the finding that cells in

the distal transition zone of the root apex, that are undergoing a preparatory phase for rapid elongation, are the most Al-sensitive (Sivaguru and Horst, 1998).

Numerous cellular functions influence cell elongation, including the regulation of Ca^{2+} at critical sites in the apoplasm. The displacement of Ca^{2+} by Al in the apoplasm could be responsible for an inhibition in root elongation (Plieth *et al.*, 1999; Rengel and Whang, 2003). Further, it appears that Al has a stronger affinity for pectin than Ca^{2+} (Blamey *et al.*, 1993) and is able to displace Ca^{2+} from the cell wall and form cross-links in the pectin material. This results in increased cell wall rigidity and reduced permeability, inhibiting cell elongation (Schmohl and Horst, 2000; Tabuchi and Matsumoto, 2001). In addition, Teraoka *et al.* (2002) suggested that inhibition of barley (*Hordeum vulgare*) root elongation could be attributed to the inhibition of cellulose synthesis by Al. Further, it has been shown that Al-induced reduction in cell wall permeability resulted in inhibited acid phosphatase and protein exudation in both cell suspension cultures and root tips of *Zea mays* (Schmohl and Horst, 2002).

In addition to the cell wall, Al also has a strong affinity for the plasma membrane (Rengel and Whang, 2003). Studies have shown that Al can disrupt the Ca^{2+}-permeable channels in the plasma membrane, Al blocks the Ca^{2+}-permeable channels and inhibits Ca^{2+} influx into the cell, thus disrupting the cytoplasmic Ca^{2+} homeostasis (Jones *et al.*, 1998; Rengel and Whang, 2003). Aluminium related changes in the cytoplasmic Ca^{2+} levels influences Ca^{2+}-dependent metabolic processes that are involved in the regulation of cell division and elongation (Rengel and Whang, 2003). Further, the interaction of Al at the plasma membrane was also shown to reduce the surface charge (Ahn *et al.*, 2001). Aluminium-induced depolarization of the plasma membrane resulted in decreased H^+-ATPase activity and an inhibition in H^+ efflux (Ahn *et al.*, 2001, 2002). Those activities are linked with the maintenance of the electrochemical gradient across the membrane and with cell expansion and division (Rengel and Whang, 2003).

Aluminium-induced lipid peroxidation of the plasma membrane has been reported for intact roots of soybean after a long treatment with Al (> 12 h) (Horst *et al.*, 1992). Although Fe

enhanced the action of Al, lipid peroxidation was not observed in less than 2 h. It was therefore suggested that Al-induced impairment of the plasma membrane was unlikely to explain inhibited soybean root elongation. Further investigations using cultured tobacco cells demonstrated the synergistic inhibition of growth by Al and Fe (Ono *et al.*, 1995). In that study Al enhanced the Fe (II) mediated peroxidation of lipids in the plasma membrane, resulting in loss of membrane integrity, reduced cell division and eventual cell death (Ikegawa *et al.*, 1998; Chang *et al.*, 1999). Aluminium, in the absence of Fe, had no apparent effect on the plasma membrane of tobacco cells (Ono *et al.*, 1995). In a recent study Yamamoto *et al.* (2001) have shown that Fe is not required to trigger Al-induced lipid peroxidation in *Pisum sativum* roots. Those authors also suggested that although lipid peroxidation was a relatively early symptom of Al toxicity, it was not linked to inhibition of root elongation and that loss of plasma membrane integrity was a late symptom caused by cracks in the root due to inhibition of root elongation.

Aluminium-induced callose (1,3 β-glucan synthase) production has often been associated with the inhibition of root growth in monocotyledonous plants (Horst, 1995). Root callose deposition was considered a good marker for Al-induced injury since in some studies it was shown to be positively related with Al accumulation and root growth inhibition (Schreiner *et al.*, 1994; Horst *et al.*, 1997; Teraoka *et al.*, 2002). Another study showed that in addition to being a marker for Al-sensitivity, callose could also be actively involved in reducing root growth. Sivaguru *et al.* (2000) proposed that in *Triticum aestivum* Al-induced callose is deposited at the plasmodesmata resulting in a blockage of symplastic intercellular transport. Cell-to-cell transport is essential for the intercellular movement of water, nutrients and other important molecules.

Growth inhibition and the swelling of roots that are exposed to Al suggest that the cytoskeleton of root cells may also be a target for Al toxicity. The plant cytoskeleton consists of two basic structural components, the microtubules (MTs) and microfilaments (MFs). The cytoplasmic MTs are recognised as important elements in plant development and they appear to participate in cell division, cell expansion and differentiation (Hepler and Hush, 1996; Hasezawa and Kumagai, 2002). It was proposed that Al alters the

cytoskeleton either by acting directly on cytoskeletal elements or indirectly through Ca^{2+}-related signalling cascades (Rengel and Whang, 2003). To date, most studies appeared to focus on the direct effects of Al on the MTs. Plant cells require a dynamic actin- and tubulin-based network for cell proliferation and differentiation. Grabski and Schindler (1995) showed that Al increased the tension and rigidity of the actin MFs in *Glycine max* cells, inhibiting cell division. Further reports linked Al exposure with a depolymerization of MTs in elongating wheat root cells (Sasaki *et al.*, 1997). Microtubule structure in elongating root cells must be maintained for whole roots to elongate normally. Aluminium-induced disruption of the MTs resulted in lateral cell swelling instead of longitudinal cell expansion in wheat root cells (Sasaki *et al.*, 1997).

Aluminium-induced effects on the cytoskeleton of cells from the inner and outer cortex of maize roots (elongation zone) were different. The MTs from the inner cortex cells were reorganised while those from the outer cortex cells were stabilised and resistant to depolymerisation by Al (Blancaflor *et al.*, 1998). The effect of Al on the cytoskeleton of maize root cells was rapid, fitted well with the time-course growth inhibition and was more pronounced for MTs than MFs. In a subsequent study on maize roots Sivaguru *et al.* (1999a) showed that Al depolymerised MTs in cells from the distal transition zone, the most Al-sensitive zone of the root tip. Others reported that the Al-induced effect on the cytoskeleton was dependent upon the growth phase of cells (Sivaguru *et al.*, 1999b; Schwarzerová *et al.*, 2002). The cortical MTs of log phase tobacco cells were depolymerised while those of stationary cells were stabilised. Recent studies also confirmed that Al depolymerises MTs in meristematic cells entering mitosis in both *Triticum turgidum* (Frantzios *et al.*, 2000) and *Allium cepa* (Dovgalyuk *et al.*, 2003). Therefore it appears that the effect of Al on the MTs is dependent upon root zone, location of root cells and the growth phase of cells.

In summary a number of theories, formulated from work with cell cultures and roots of whole plants, have been proposed in order to explain the cause of Al toxicity in plants (Table 1.2). Aluminium has been shown to inhibit cell elongation, cell division and ultimately suppress root growth through disruptions in the functioning of the cell wall,

plasma membrane, nucleus, mitochondria and plant cytoskeleton. Cells in the distal transition zone of the root were identified as the primary target for Al (Sivaguru and Horst, 1998). Aluminium was shown to depolymerise MTs in these cells, thus inhibiting further development (i.e. elongation). Aluminium-induced inhibition of root elongation was shown within 1 h, at which stage Al was found to stabilise the MTs in the outer cortex cells but was not detected in the inner cortex cells of the root (Blancaflor *et al.*, 1998). It may therefore be concluded that during the initial stages of Al toxicity (< 1 h) root elongation is probably inhibited through direct and indirect Al-induced effects. The former would include stabilisation or depolymerisation of the MTs and the reduced permeability of the cell wall. Alterations in the plant cytoskeleton and cell wall can result in a disturbance in the cytoplasmic Ca^{2+} homeostasis (Rengel and Whang, 2003), these changes in Ca^{2+} levels can be communicated to adjacent cells and represent an indirect Al-induced effect.

1.6 Al Resistance

The widely held view is that Al toxicity arises from Al interactions with several different processes within the root (as discussed above). It is, therefore, assumed that Al resistance is going to be equally complex, with plants having a variety of different Al resistance mechanisms (Taylor, 1995; Kochian, 1995; De la Fuente-Martinez and Herrera-Estrella, 1999; Matsumoto *et al.*, 2001).

There is considerable variation among plant species in their response to Al, with some showing severe growth inhibition while others remain unaffected. It appears that plants avoid Al damage by either excluding Al (i.e. Al entry into root cells is slowed down or prevented) or by tolerating Al (i.e. Al is detoxified in the symplasm) (Taylor, 1995; Kochian, 1995). Those that exclude Al from the root cells are therefore Al-resistant (Al-R) and those that tolerate Al in the symplasm, Al-tolerant (Al-T) (Kochian, 1995). The term Al-T has been used inconsistently in the literature to refer to plants that employ mechanisms of Al exclusion which are, in fact, Al-R. In this work the general term Al-R is used, with the descriptor Al-T being only used when used and defined by other authors.

Chelation of Al in the cytoplasm by organic acids, proteins or other organic ligands and compartmentation of Al into vacuoles are examples of symplasmic mechanisms of Al tolerance (Taylor, 1995; Kochian, 1995). Mechanisms that enable the plant to avoid Al uptake into cells include exudation of Al chelators (e.g. organic acids) into the rhizosphere, formation of a plant-induced pH barrier in the rhizosphere and immobilization of Al in the cell wall (Kochian, 1995; Matsumoto, 2000). To date evidence supporting the existence of most of those mechanisms is available if limited. However, the majority of research on Al resistance has focused on the release of organic acids in response to Al. Table 1.3 summarises some of the main experimental studies on the mechanisms of Al resistance.

Although the mechanisms responsible for Al-induced inhibition of root elongation are complex, it is known that Al injury results generally from the binding of free Al ions to root cell components (Ma, 2000; Čiamporová, 2002). Therefore, if a ligand binds strongly to Al then the activity of free Al ions would be reduced. Such ligands are represented by the organic acids (citric, oxalic, malic, tartaric, salicyclic and malonic), all of which form a stable complex with Al (Ma, 2000). Citrate is one of the strongest Al-detoxifying agents with the resulting Al-citrate complex having a high stability constant (Hue *et al.*, 1986). Equimolar amounts of citrate can detoxify Al (1:1), but three times more oxalic and six to eight times more malic acid is required to achieve the same effect. For this reason many studies have linked resistance to Al with the exudation of citrate (Pellet *et al.*, 1995; Mugai *et al.*, 2000; Yang *et al.*, 2000; Kollmeier *et al.*, 2001).

Aluminium can trigger the release of organic acids, which are exuded specifically at the root tip and the Al-R genotypes release more organic acid than the Al-S genotypes (Jones, 1998; Ma, 2000; Kochian *et al.*, 2002). Delhaize *et al.* (1993b) reported that Al resistance in wheat (*Triticum aestivum*) was associated with increased malate efflux. It appears that the Al-induced release of malate is restricted to wheat since malate efflux has not been implicated in the differential resistance of any other Poaceae (Parker and Pedler, 1998; Jones, 1998; Ma, 2000). Although Li *et al.* (2002) showed that Al-T *Secale cereale* seedlings produced both malate and citrate in response to Al, citrate was found to be responsible for the increased tolerance to Al and not malate. The release of citrate in

Table 1.3 Proposed mechanisms of aluminium resistance

Species	Plant material	Class of Al-R	Mechanism of resistance	Plant response	Reference
Triticum aestivum	Al-S and Al-T seedlings	Al exclusion	Exudation of Al-chelating ligand (malate) into rhizosphere	Al-T exuded 5-10x more malate than Al-S, reduced RGI in Al-T plants	Delhaize *et al.*, 1993b; Ryan *et al.*, 1995
Zea mays	Al-S and Al-T seedlings	Al exclusion	Exudation of Al-chelating ligand (citrate) into rhizosphere	Al-triggered release of citrate at the root apex, reduced RGI in Al-T plants	Pellet *et al.*, 1995; Jorge and Arruda, 1997
Phaseolus vulgaris	Al-S and Al-T seedlings	Al exclusion	Exudation of Al-chelating ligand (citrate) into rhizosphere	increased citrate synthase activity, Al-T produced more citrate than Al-S	Mugai *et al.*, 2000
Glycine max	Al-S and Al-T seedlings	Al exclusion	Exudation of Al-chelating ligand (citrate) into rhizosphere	Al-T exuded more citrate than Al-S, RGI reduced in Al-T	Yang *et al.*, 2000
Secale cereale	Al-S and Al-T seedlings	Al exclusion	Exudation of Al-chelating ligands (citrate and malate) into rhizosphere	Al-induced specific release of citrate by Al-T root tips, RGI reduced in Al-T	Li *et al.*, 2002
Triticum aestivum	Al-S and Al-T protoplasts	Al exclusion	Exudation of Al-chelating ligand (malate) into rhizosphere	Malate efflux through Al-induced anion channel, more channels in Al-T than Al-S	Zhang *et al.*, 2001
Zea mays	Al-S and Al-R protoplasts	Al exclusion	Exudation of Al-chelating ligand (citrate) into rhizosphere	citrate efflux through anion channels in cells from DTZ of root apex	Kollmeier *et al.*, 2001
Nicotiana tabacum	wildtype and transgenic plants	Al exclusion	Exudation of Al-chelating ligand (citrate) into rhizosphere	overproduction of citrate resulted in Al-T in transgenic plants	De la Fuente *et al.*, 1997

Table 1.3 contd.

Species	Plant material	Class of Al-R	Mechanism of resistance	Plant response	Reference
Daucus carota	cell suspension	Al exclusion	Exudation of citrate into growth medium, overexpression of CS gene resulted in increased citrate efflux	-	Koyama *et al.*, 1999
Triticum aestivum	Al-S and Al-T seedlings	Al exclusion	Exudation of root exudate polypeptides	Al-T releases more REP than Al-S, reduced RGI	Basu *et al.*, 1994; Basu *et al.*, 1999
Triticum aestivum	Al-S and Al-T seedlings	Al exclusion	Increased rhizosphere pH	Al-T roots resist acidification in the presence of Al	Taylor and Foy, 1985
Oryza sativa	Al-S and Al-T seedlings	Al exclusion	Increased rhizosphere pH	Al-T cultivars accumulated less Al in their foliage than Al-S	Sivaguru and Paliwal, 1993
Arabidopsis thaliana	wildtype, Al-R seedlings	Al exclusion	Increased rhizosphere pH	Al-R increased root surface pH, reduced RGI	Degenhardt *et al.*, 1998
Phaseolus vulgaris	Al-S and Al-R seedlings	Al exclusion	Exudation of mucilage by root border cells	Mucilage binds Al and reduces Al solubility, reduced RGI	Miyasaka and Hawes, 2001
Fagopyrum esculentum	Al-R seedlings	Al exclusion + Al tolerance	Exudation of oxalate by root tip Symplasmic Al-detoxification in leaves	Al-induced release of chelating agent by roots and internal Al chelation in cell sap of leaves	Ma *et al.*, 1998

Table 1.3 contd.

Species	Plant material	Class of Al-R	Mechanism of resistance	Plant response	Reference
Zea mays	Al-T seedlings	Al exclusion + Al tolerance	Exudation of Al-chelating ligand (citrate), symplasmic Al-detoxification (inc. citrate levels)	Al-induced citrate exudation followed by increased internal citrate content	Piñeros *et al.*, 2002
Zea mays	Al-S and Al-R seedlings	Al exclusion + Al tolerance	Lysigeny of Al-hyperaccumulator cells, Al accumulated in vacuoles	reduced RGI in Al-R plants	Vazquez, 2002
Triticum aestivum	Al-S and Al-T seedlings	Al exclusion	Exudation of Al-chelating ligands (malate + phosphate), increased rhizosphere pH	Al^{3+} activity reduced, reduced RGI	Pellet *et al.*, 1997
Brachiara decumbens *B. ruziziensis*	Al-R seedlings	Al exclusion	Exudation of organic acids (citrate, malate, oxalate), increased rhizosphere pH, and alternative mechanisms (to be determined)	reduced RGI in *B. decumbens*	Wenzl *et al.*, 2001

Al-S = Aluminium-sensitive, Al-R = Al-resistant, Al-T = Al-tolerant, RGI = root growth inhibition, DTZ = distal transition zone, CS = citrate synthase, REP = root exudate polypeptide

response to Al has been reported for, amongst others, *Zea mays* (Pellet *et al.*, 1995), *Cassia tora* (Ma *et al.*, 1997), *Daucus carota* (Koyama *et al.*, 1999), *Brassica napus* (Luo *et al.*, 1999), *Phaseolus vulgaris* (Mugai *et al.*, 2000), *Glycine max* (Yang *et al.*, 2000) and *Nicotiana tabacum* (Delhaize *et al.*, 2003).

A primary mechanism in this organic acid exudation is that Al has been shown to induce the activation of anion channels facilitating the exudation of organic acids. In wheat, a study using protoplasts showed differences in Al^{3+}-induced malate efflux between Al-T and Al-S genotypes. The Al-T genotype had a higher capacity to activate malate permeable channels for sustained malate release than the Al-S (Li *et al.*, 2000a; Zhang *et al.*, 2001). In maize, Al appeared to stimulate the activation of anion channels for citrate release in cells from the distal transition zone of the root apex. Such activation of anion channels was not observed in the presence or absence of Al in cells from the elongation zone (Kollmeier *et al.*, 2001). Aluminium-induced anion channels have been localised in the root-cell plasma membrane (Piñeros and Kochain, 2001). To date, experimental evidence has established that Al induces the release of organic acids, the Al-R genotype releases more organic acid than the Al-S genotype and anion channels facilitate organic acid exudation. However, there appear to be conflicting reports as to whether increased organic acid levels in root cells are linked to an increased rate of efflux. Some authors attributed Al tolerance to increased citrate synthase activity, increased internal citrate levels and increased efflux in response to Al (Mugai *et al.*, 2000; Li *et al.*, 2000a), while others found no link between increased internal citrate content and efflux (Watt and Evans, 1999; Delhaize *et al.*, 2003).

Despite the conflicting evidence regarding the link between increased internal citrate levels and Al resistance, there are studies in which the level of resistance to Al has been manipulated through increased citrate efflux (De la Fuente *et al.*, 1997). The *Pseudomonas aeruginosa* citrate synthase (CS) gene was expressed in tobacco and the resulting transgenic (Al-T) plants showed a ten-fold increase in internal citrate levels and a four-fold increase in efflux. Similarly, Koyama *et al.* (1999) over-expressed the CS gene in carrot cell lines and showed that improved growth in the Al-phosphate medium was linked with increased citrate efflux. To date, there is strong experimental evidence that links Al-induced

organic acid release with Al resistance (De la Fuente *et al.*, 1997; Yang *et al.*, 2000; Zhang *et al.*, 2001; Li *et al.*, 2002). However, the physiological mechanisms involved in the regulation of this Al-induced response is still a question for debate (Jones, 1998). Some authors questioned whether the levels of organic acids released were adequate to achieve the level of Al resistance required. In fact, Parker and Pedler (1998) suggested that the amount of malate released in wheat was not sufficient to prevent Al damage. They proposed that the exudation of malate together with other mechanisms of Al exclusion were responsible for preventing Al inhibited root growth in wheat, therefore, concluding that there was a multifaceted approach to Al resistance in wheat.

There is limited evidence in the literature, other than organic acid exudation, in support of mechanisms for Al chelation or exclusion. In one study, Basu *et al.* (1994, 1999) suggested the release of high molecular mass root polypeptides that are able to bind Al and reduce entry into root cells. Aluminium-tolerant wheat genotypes were shown to exude significantly more polypeptides than Al-S. Other authors proposed alkalization of the rhizosphere as means to exclude Al from root cells since even small increases in pH may result in large decreases in Al solubility (Kinraide, 1997). Although early studies reported Al-induced increases in rhizosphere pH of wheat (Taylor and Foy, 1985) and rice (Sivaguru and Paliwal, 1993), they were not convincing since pH measurements were made in bulk nutrient solution. To date the most convincing study for Al exclusion through increased rhizosphere pH was proposed for *Arabidopsis thaliana* by Degenhardt *et al.* (1998). Those authors studied resistance to Al by comparing the responses of two *A. thaliana* mutants (*alr-104*, *alr-128*) and the wildtype to Al. Both mutants were resistant to Al by adopting two different mechanisms of Al exclusion. The first mutant (*alr-104*) reduced Al availability in the growth medium through increased rhizosphere pH, achieved by increased H^+ influx at the root apex. In that study no significant pH difference was found between the mutant and wildtype in the absence of Al. However, when roots of *alr-104* were exposed to Al the surface pH increased to 4.53 whereas that of the wildtype remained at 4.39. Further it was shown that an increase in rhizosphere pH of 0.1 unit resulted in increased Al resistance. The second mutant reduced Al entry into root cells through an increase in organic acid (citrate and malate) release (Larsen *et al.*, 1998).

Another possible mechanism of Al avoidance is the adsorption of Al by negatively charged root mucilage, which prevents the migration of Al into the root cells. Root apices of most plants exude a mucilaginous substance (polysaccharides which contain mainly uronic acid) that serves to protect the growing root tip. Mucilage has a high affinity for Al and is therefore able to reduce Al solubility and entry into root cells (Horst *et al.*, 1982). In that study mucilage exuded by the Al-T cultivar of cowpea bound more than half of the total Al content at the root tip and removal of the mucilaginous layer resulted in root growth inhibition. Li *et al.* (2000b) also reported that Al binds strongly to maize mucilage. However, in that study, exudation of mucilage alone did not protect roots from Al injury. In another report Miyasaka and Hawes (2001) showed that Al-R *Phaseolus vulgaris* root border cells exuded more mucilage than the Al-S cells. They suggested that increased release of Al-binding mucilage by border cells could play a role in protecting root tips from Al-induced injury.

There are many reports linking Al-resistance with Al avoidance (i.e. excluding Al from root cells) and although mechanisms for symplasmic Al-detoxification have been proposed there is limited evidence to support them. Ma *et al.* (1998) showed that Al injury was prevented in *Fagopyrum esculentum* roots through the transport of Al to the leaves where it was chelated with oxalate in the cell sap. Piñeros *et al.* (2002) reported that Al-T maize seedlings could have two possible mechanisms of Al-resistance. The first, Al-induced citrate exudation resulting in Al chelation in the rhizosphere and the second increased internal citrate levels indicating possible symplasmic Al-detoxification. Recently Vazquez (2002) proposed that Al-resistance in certain maize genotypes was achieved through both Al exclusion and (internal) tolerance. Ultrastructural studies revealed that Al-injury was prevented in maize root cells through Al-chelation in vacuoles in the symplasm (Vazquez, 2002). In addition, it appeared that certain cells were designated to hyperaccumulate Al and degenerate to form a lysigenic cavity. It seemed that certain cells were 'sacrificed' in order to contain Al in lysigenic cavities of the root tissue, thereby excluding Al from other meristematic root cells and thus maintaining overall root growth (Vazquez, 2002).

In their important reviews Taylor (1995) and Kochian (1995) proposed that since plants are complex multigenic systems, it is unlikely that a single resistance mechanism can explain Al resistance. They reported that resistance to Al is most probably mediated by an integrated suite of adaptive responses. Although in most species the majority of researchers have focused on a single mechanism for Al resistance, some have considered multiple exclusion mechanisms (Pellet *et al.*, 1997; Ma *et al.*, 1998; Wenzl *et al.*, 2001; Vazquez, 2002; Piñeros *et al.*, 2002). Pellet *et al.* (1997) suggested that tolerance to Al in wheat could be explained by several mechanisms of Al exclusion. They showed that Al-T wheat avoided Al injury through the release of Al chelating ligands (malate and phosphate) as well as an increase in rhizosphere pH. In another study, Wenzl *et al.* (2001) reported that the current model for Al resistance, based on external detoxification of Al by chelating ligands or alkalization of the rhizosphere, could not adequately explain the high Al resistance in signalgrass (*Brachiaria decumbens*). Those authors proposed additional mechanisms of Al resistance for which there is limited evidence in the literature; low permeability of the plasma membrane to Al, active Al extrusion from the cell combined with symplastic Al tolerance. More recently Piñeros *et al.* (2002) reported that resistance to Al is genetically and physiologically complex with multiple mechanisms of Al exclusion responsible for protecting maize roots from Al injury. Those authors suggested an initial weak response to Al (the release of malate and phosphate) that activates anion channels for the specific release of citrate, followed by increased internal production of organic acids for symplasmic Al detoxification.

1.7 *Cynodon dactylon*

Grasses are the most widespread plants on earth. Of some 10 000 species occurring world-wide, 967 occur in South Africa (Gibbs Russel *et al.*, 1990). The Poaceae family is extremely important for global food production and includes all the cereal crops and 75% of cultivated forage species (Wang *et al.*, 2001). The most intensively used forage and turf grasses include fescues (*Festuca* spp), ryegrasses (*Lolium* spp), bentgrasses (*Agrostis* spp), bluegrasses (*Poa* spp), bromegrasses (*Bromus* spp), orchardgrasses (*Dactylis* spp), switch- and guineagrasses (*Panicum* spp) and bermudagrasses (*Cynodon* spp).

The *Cynodon* genus comprises of nine species and 10 varieties and contains grasses of economic importance for livestock herbage, turf and soil stabilization (Assefa *et al.*, 1999). *Cynodon dactylon* (L.) Pers. is the most conspicuous and successful member of the genus and is ubiquitous through the tropics and subtropics (Harlan and de Wet, 1969 ; Assefa *et al.*, 1999). It is commonly referred to as bermudagrass (Table 1.4, Figure 1.1) and is regarded as one of the most widely distributed and successful grass species in the world (Assefa *et al.*, 1999). It is a creeping perennial and can form a thick mat over the soil surface by means of stolons and rhizomes which is why it is considered an important turf grass (e.g. for the prevention of soil erosion) (Feagley *et al.*, 1994 ; Taliaferro, 1995 ; Chapman, 1996). *Cynodon dactylon* is a rhizomatous perennial grass that is propagated vegetatively mainly through stolon fragmentation (Fernandez, 2003).

Table 1.4 Classification of *Cynodon dactylon*

Taxonomic affinity	Bermudagrass
Family	Poaceae
Subfamily	Chloridoideae
Tribe	Cynodonteae
Subtribe	Chloridinae
Genus	*Cynodon*
Species	*dactylon*

Chapman, 1996

Cynodon dactylon flowers from September to March carrying a terminal digitate inflorescence (Figure 1.1). The leaf blade is narrow, ending in a sharp point and a short membrane ringed with hairs forms the ligule (van Oudtshoorn, 1992; Chapman, 1996). This grass grows in most soil types and in South Africa it has been found on soils from moist-clays to dry-sandy soils (Bredenkamp and van Rooyen, 1996). It occurs mostly in disturbed areas, overgrazed fields, uncultivated lands, road verges and river embankments (van Oudtshoorn, 1992).

Figure 1.1 Mature *Cynodon dactylon* plant with terminal digitate inflorescence. Scale bar = 20 mm.

Common and African-type bermudagrasses constitute an enormous genetic pool that encompasses numerous variant types and naturally occurring hybrids (Taliaferro, 1995; Caetano-Anolles *et al.*, 1997). Reported chromosome numbers range from 18 (2n = 2x) to 54 (2n = 6x) (Taliaferro *et al.*, 1997). Natural and induced hybridisation of tetraploid *C. dactylon* L. Pers. var. *dactylon* and *C. transvaalensis* Burt-Davy plants provided triploid (2n = 3x = 27) cytotypes that are widely used as turf (Taliaferro, 1995; Taliaferro *et al.*, 1997; Caetano-Anollés, 1997). A hexaploid (2n = 6x = 54) *C. dactylon* var. *dactylon* was crossed with *C. barberi* to produce a pentaploid (2n = 5x = 45) hybrid; hexaploid *Cynodon* plants occur very rarely (Taliaferro *et al.*, 1997). Many hybrid bermudagrass cultivars are sterile and can only be vegetatively propagated (Zhang *et al.*, 2003).

The 'cosmopolitan' *C. dactylon* var. *dactylon* is enormously diverse with plants extremely variable in growth, ranging from very small fine turfy types to robust course types (Taliaferro, 1995). Harlan and de Wet (1969) separate the taxon into 3 races (tropical, temperate and seleucidus) based on appearance, adaptation and geographical distribution. Plants of the tropical race are generally short and well adapated to the wet, infertile and acid soils of the tropics. Temperate race plants are adapted to the cold (-12 ± 3 °C) and are also generally short in appearance but denser than tropical types. Those plants require soils with higher pH and nutrient status than the tropical types. Seleucidus race plants are distributed in the region of the original Seleucid empire (Pakistan to Turkey). Those grasses are markedly different from the other two races with typically taller, very coarse, robust plants bearing stout stolons and rhizomes with short internodes (Harlan *et al.*, 1970). *Cynodon dactylon* is therefore regarded as one of the most dynamic and aggressive species in the world.

Fuls and Bosch (1990) collected 140 *C. dactylon* plants from six different climatic regions in South Africa, and areas characterised by low soil nutrient status, low soil pH (pH 4.4 – 5.4), disturbed habitat and low annual rainfall (151 – 685 mm). In that study all the genotypes tested were resistant to low pH and certain genotypes performed well in dry areas, some grew well in disturbed sites while others preferred soils with low nutrient levels. Those authors found that none of the genotypes collected performed well under all the tested environmental conditions. Thus it seemed that those *C. dactylon* populations were adapted to the specific environmental stresses that were probably present in the areas from which they were collected.

Bermudagrass has been selected for the revegetation of large surface-mined areas in the southern United States since many varieties of this grass are adapted to those areas (infertile soils, low pH) (Feagley *et al.*, 1994; Haby, 2002). Mine soils from those areas are characterised by low organic C, low pH (5.5) and elevated metal content (Al, Fe, Cu, Mn and Zn). In that study, the revegetation of mine waste with *C. dactylon* was facilitated by the use of papermill sludge and fertilizer (Feagley *et al.*, 1994). In another study, *C. dactylon* was selected for the revegetation of coal ash waste in semi-arid regions of

Zimbabwe (Piha *et al.*, 1995). Those mine tailings were nutrient deficient (P, K, Ca, Mg, S and micronutrients), high in pH (8.6) and contained an elevated aluminate content ($Al(OH_4^-)$, 43.8 ± 4.3 μg g^{-1}). *Cynodon dactylon* vegetation cover was established on partially ameliorated mine tailings (minimal additions of: N, P, K, S, Ca, Mg and micronutrients) (Piha *et al.*, 1995). In that study, the *C. dactylon* varieties selected appeared to have low nutritional requirements and a tolerance to increased Al levels and high pH. *Cynodon dactylon* has also been used to revegetate and stabilise Zn and Pb mine tailings in China and in that study this grass outperformed other grasses (*Agropyron elongatum* and *Lolium multiflorum*) forming an effective vegetative cover over the tailings (Ye *et al.*, 2000). Those mine tailings were also nutrient deficient, high in sulphides and low in pH. It appears, therefore, that the 'cosmopolitan' *C. dactylon* is a genetically diverse grass that can adapt to grow under several different kinds of environmental stresses (i.e. nutrient deficient soils; high metal (in particular Al, Pb, Zn) toxicity and low and high pH). Although varieties of *C. dactylon* have been found growing in areas with low pH (Fuls and Bosch, 1990 ; Feagley *et al.*, 1994 ; Ye *et al.*, 2000 ; Haby, 2002) and high Al (Feagley *et al.*, 1994 ; Piha *et al.*, 1995), to date there appears to be no work that focuses on the selection of Al-R *C. dactylon* genotypes.

Small populations of *C. dactylon* were found growing on gold mine tailings on the Witwatersrand (Gauteng Province) in South Africa. Those tailings represent infertile soil that is particularly low in pH (2.7 – 5.6) with high exchangeable Al (162 – 5508 mg kg^{-1}) (Cooke *et al.*, 1995). *Cynodon dactylon* plants were collected from a number of these populations. It was assumed that populations of *C. dactylon* found growing on the tailings could have developed a resistance to Al and a low pH, making such Al-R plants suitable for the revegetation of gold tailings dams in South Africa. A number of plants collected from the gold mine tailings did not flower or produce seed and these are similar to the hybrid bermudgrasses that can only be propagated vegetatively (Taliaferro, 1995; Chapman, 1996; Zhang *et al.*, 2003).

1.8 *In vitro* propagation and its applications

In vitro propagation or micropropagation provides an alternative means of regenerating plants vegetatively, whereby a piece of leaf, stem or root can be manipulated (via organogenesis or embryogenesis) under *in vitro* conditions to produce many clonal replicates of the original parent plant (George, 1996; Barnum, 1998). In addition to the rapid mass propagation of selected genotypes, this approach offers the means to produce disease- and virus- free plants, generate new varieties through somaclonal variation, conserve the germplasm of desired species and monitor various physiological and biochemical responses in cell suspension cultures (George, 1996; Barnum, 1998). Another advantage is that the *in vitro* propagation system provides a suitable framework for the incorporation of a foreign piece of DNA into the genome of the plant cell, which is then regenerated into a mature transgenic plant (Vasil and Vasil, 1994). Genetic engineered plants are thought to be revolutionising the agricultural industry with new high yielding crop varieties that are tolerant to various biotic and abiotic environmental stresses (Hansen and Wright, 1999; Jauhar, 2001; Kern, 2002; Williamson, 2002; Potenza *et al.*, 2004). This aspect of plant biotechnology is highly controversial (Daniell, 1999; Trewavas, 1999; Jauhar, 2001; Sharma *et al.*, 2002; Williamson, 2002; Giovanetti, 2003; Nap *et al.*, 2003; Sonnewald, 2003; Miki and McHugh, 2004; Potenza *et al.*, 2004) and further details are not provided here since the production of transgenic plants is beyond the scope of the present study.

In vitro propagation protocols have been published for *C. dactylon*. In those studies the immature inflorescence has been used to establish somatic embryogenesis regeneration protocols (Ahn *et al.*, 1985; Artunduaga *et al.*, 1988; Chaudhury and Qu, 2000; Li and Qu, 2002). In those manipulations, somatic cells of the young unemerged flower are stimulated to de-differentiate under suitable *in vitro* conditions to produce callus, an unorganised mass of rapidly dividing cells (Wang *et al.*, 2001). Callus cells later differentiate into somatic embryos that mature and regenerate the whole plant. However, this *in vitro* regeneration protocol based on the immature inflorescence was not suitable in the present study since a number of *C. dactylon* plants collected from the gold tailings did not flower.

Micropropagation protocols using a vegetative explant source were therefore established in this work, details of which are presented in chapter 2.

In addition to rapid clonal propagation, *in vitro* systems may also be used for screening plants for specific environmental stresses, such as high Al and low pH. Such an approach has been reported for various species, using both cell suspension (Ojima and Ohira, 1983; Conner and Meredith, 1985b; Yamamoto *et al.*, 1994) and callus cultures (Smith *et al.*, 1983; Parrot and Bouton, 1990; Espino *et al.*, 1998). A review of the literature has revealed that the *in vitro* screening of *C. dactylon* cultures for resistance to Al has not been considered. However, such a cellular approach has been favoured in other studies to select plants that are resistant to metals other than Al. Those studies include the *in vitro* selection and regeneration of copper-tolerant *Nicotiana tabacum* (Gori *et al.*, 1998), zinc- and manganese-tolerant *Brassica* spp. (Rout *et al.*, 1999) and chromium- and nickel-tolerant *Echinochloa colona* (Samantaray *et al.*, 2001). Similar *in vitro* techniques have been used in the selection and regeneration of salt tolerant *Triticum aestivum* (Barakat and Abdel-Latif, 1996), *Brassica juncea* (Gangopadhyay *et al.*, 1997), *Cymbopogon martinii* (Patnaik and Debata, 1997), *Oryza sativa* (Basu *et al.*, 1997) and *Solanum tuberosum* (Ochatt *et al.*, 1999).

Despite the considerable progress made over the past decade with regard to mechanisms of Al toxicity and resistance, the molecular basis of Al tolerance in plants is still far from being completely understood (Drummond *et al.*, 2001; Milla *et al.*, 2002). A number of genes expressed specifically as a result of Al stress have been identified in the cereal crops (Snowden and Gardner, 1993; Snowden *et al.*, 1995; Sibov *et al.*, 1999; Milla *et al.*, 2002; Sasaki *et al.*, 2002). Those investigations revealed that metal toxicity induces the expression of a diverse array of genes, including several that are involved in general plant stress. A total of 13 novel genes involved in cell elongation and division, oxidative stress, iron metabolism and other cellular mechanisms appeared to be regulated by Al stress in rye (Milla *et al.*, 2002). Further, in a recent study on sugarcane it was reported that the genic fragments isolated from the root tips were responsive to both Al^{3+} and oxidative stress (Watt, 2003). It has been suggested, therefore, that common gene induction mechanisms

exist for different stresses. The biological role of Al-induced genes in Al stress is still unclear and further research towards this end is needed. As mentioned earlier, gene transfer systems in plants are dependent upon an efficient *in vitro* regeneration protocol. This technology, therefore, enables for the conversion of Al-S plants into an Al-R state. For example, genes identified during Al-stress in the resistant varieties may be isolated and transferred into sensitive genotypes.

1.9 Aims and Objectives of this study

1.9.1 Research aims

Current research has shown that Al^{3+} limits plant growth by inhibiting root elongation. Although a number of theories have been formulated in order to explain the inhibitory effects of Al^{3+}, the primary mechanism and initial targets of Al^{3+} are still unclear. The present study, therefore, considered an alternative approach to investigating Al toxicity and resistance. This involved the exposure of a group of actively dividing meristematic cells (callus) to Al^{3+} *in vitro*. The focus of this study was to assess the feasibility of using *in vitro* meristematic callus cells to investigate mechanisms of Al^{3+} toxicity and resistance.

Cynodon dactylon was selected as the plant material for this investigation since small populations of potentially Al-resistant genotypes of this grass were found growing on gold tailings. This study also investigated the potential of using differences in callus growth to identify Al-resistant genotypes since such plants were required for the revegetation of the tailings. As vegetative propagation of *C. dactylon* is slow, labour intensive and limited by stolon production. However, large numbers of Al-R *C. dactylon* genotypes are required for the establishment of a vegetation cover on the tailings, therefore, additional means of multiplying these desired *C. dactylon* genotypes were also investigated.

1.9.2 Thesis structure

Vegetative explants were used to establish *in vitro* regeneration protocols for *C. dactylon* (Chapter 2). Plant regeneration was achieved via the indirect somatic embryogenesis and direct organogenesis morphogenic pathways. This approach facilitated the initiation of callus from young leaves, the regeneration of whole plants from callus and provided an

alternate means for the conventional method of vegetative propagation of *C. dactylon* via stolons.

The response of meristematic callus cells to Al^{3+} was investigated. As callus growth required a nutrient medium containing both an available supply of the phytotoxic Al ion (Al^{3+}) as well as all the essential macro- and micro-nutrients required for cell division, a chemical speciation model was used to predict suitable medium modifications that were used to develop an Al^{3+}-screening medium (Chapter 3). Calli of three *C. dactylon* genotypes were exposed to Al^{3+} and the different responses of these genotypes in the presence of Al^{3+} facilitated the identification of an Al-S and Al-R genotype. Further studies were conducted to investigate possible reasons for the greater inhibitory effect of Al^{3+} on the Al-S than the Al-R genotype (Chapter 4). These included the establishment of cell counts and the localisation and measurement of Al within the callus cells. Aluminium resistance strategies published for whole plants in other studies were also investigated in the present work.

Lastly, the merits of using an alternate approach for the investigation of mechanisms of Al^{3+} toxicity and resistance was considered (Chapter 5).

CHAPTER 2: ESTABLISHMENT OF *IN VITRO* REGENERATION PROTOCOLS

2.1 Introduction

The establishment of *in vitro* regeneration systems has played a significant role in the biotechnological improvement of grasses and cereals (Vasil and Vasil, 1994). Although impressive progress has been achieved towards developing efficient plant regeneration systems for all the major cereal crops, forage and turf grasses have received far less attention (Krishnaraj and Vasil, 1995; Wang *et al.*, 2001). In this study, *in vitro* regeneration systems were investigated for *C. dactylon* since such systems offer an alternative means for the vegetative propagation of this grass. Furthermore the *in vitro* system could be adapted to monitor the physiological and biochemical responses of *C. dactylon* to a particular stress (i.e. Al^{3+} toxicity). This chapter presents a summary of *in vitro* work in the Poaceae, reports on the establishment of organogenesis and somatic embryogenesis regeneration protocols for *C. dactylon* and evaluates them with those established for other grasses and cereals.

Grasses, like most plants, present a propensity for asexual or vegetative propagation (Chapman, 1996; Fernandez, 2003). This capacity is fully expressed in plant cells and tissues and has been manipulated *in vitro* for plant propagation. *In vitro* regeneration can be accomplished through four basic methods: 1) axillary shoot proliferation, 2) node culture, 3) *de novo* formation of adventitious shoots and 4) nonzygotic embryogenesis (Kane, 2000). The first three methods follow the organogenesis morphogenic pathway and nonzygotic (usually somatic) embryogenesis makes up the fourth. Plant regeneration via organogenesis is achieved through shoot development that is followed by rooting, while somatic embryogenesis results in the formation of bipolar embryos that contain both root and shoot meristems. Although both morphogenic routes have been used for the *in vitro* regeneration of the grasses, the somatic embryogenesis approach appeared to be preferred by most authors (Wang *et al.*, 2001).

Somatic cells contain all the genetic information (totipotent) necessary for plant regeneration. Isolated somatic cells can develop normally into embryos or stimulated into developing plant organs. It is not known what determines a somatic cell to follow either the embryogenic or organogenic morphogenic developmental route (Joy and

Thorpe, 1999). It has been proposed, however, that plant growth regulators and stress (induced by wounding or changes to the cellular environment) play a central role in causing genetic, metabolic and physiological reprogramming, resulting in either embryogenic or organogenic competence (Joy and Thorpe, 1999; von Arnold *et al.*, 2002; Fehér *et al.*, 2003).

Organogenesis is the formation of individual organs, such as shoots or roots (Hicks, 1980; Phillips and Hubstenberger, 1995). Two organogenic events comprise a common approach to *in vitro* plant propagation. The first is to regenerate multiple shoot meristems or to induce existing ones to break dormancy and produce shoots. The second involves the induction of *de novo* root meristem (Schwarz and Beaty, 2000). The *in vitro* production of plants via organogenesis can also go through an intervening callus phase. This organogenic callus produces shoots that are rooted to give plantlets. Direct shoot production from lateral buds of nodes avoids cycles of de-differentiation and re-differentiation that occur with regeneration from callus. The presence of the proliferating callus stage (indirect organogenesis) has the potential to interfere with ongoing molecular events that drive *de novo* organ production or induce genetic variations (Schwarz and Beaty, 2000). Therefore, plantlet regeneration via node culture (direct organogenesis) is an effective and efficient method for clonal propagation (Kane, 2000).

Table 2.1 provides a summary of some of the *in vitro* regeneration protocols that have been published for a selection of grasses and cereals. The majority of the literature on grass micropropagation involves the use of somatic embryogenesis and not organogenesis protocols for plant regeneration (Wang *et al.*, 2001). In early studies, the latter route was pursued since it is the simplest type of *in vitro* regeneration and it offered clonal regenerants in a relatively short time (Jullien and Tran Thanh Van, 1994; Alexandrova *et al.*, 1996a; Flachsland *et al.*, 1997). Further, some species were found to be recalcitrant to the somatic embryogenesis efforts for plant regeneration and in those cases the organogenesis approach provides an alternative regenerative route for *in vitro* propagation. Also, the organogenesis culture system offers the means to eradicate pathogens from desired regenerants (Kane, 2000) and provides an *in vitro* source of explants for other *in vitro* cultures (Alexandrova *et al.*, 1996b).

Table 2.1 A summary of some published studies on micropropagation in the Poaceae

Species	Route	Explant	Results	Culture medium composition					References
				Nutrients	Sugar	Amino acid	Plant hormones	Other additives	
Grasses									
Agropyron spp. (Wheatgrass)	O	young inflorescence	callus & shoot formation	LS	sucrose	-	2,4-D + kin	-	Lo *et al.*, 1980
		shoots	shoots rooted	LS	sucrose	-	-	-	
Agrostis alba (Red top)	SE	mature seeds	callus formation	MS	sucrose	pro + thio	2,4-D	-	Shetty & Asano, 1991
		callus	plant regeneration	MS	sucrose	-	-	-	
Alopecurus arundinaceus (Creeping foxtail)	O	young inflorescence	callus & shoot formation	LS	sucrose	-	2,4-D + kin	-	Lo *et al.*, 1980
		shoots	shoots rooted	LS	sucrose	-	-	-	
Andropogon gerardii (Big bluestem)	O	young inflorescence	callus & shoot formation	LS	sucrose	-	2,4-D + kin	-	Chen *et al.*, 1977
		shoots	shoots rooted	LS	sucrose	-	-	-	
Bambusa glaucescens (Bamboo)	O	nodal segment	axillary bud elongation & rooting	MS	sucrose	gly	NAA	-	Jullien & Tran Thanh Van, 1994
	SE	mature caryopse	callus	MS	sucrose	-	2,4-D	-	Saxena and Dhawan, 1999
		callus	plant regeneration	MS	sucrose	-	NAA + kin	-	
Bromus inermis (Smooth bromegrass)	O	young inflorescence	callus & shoot formation	LS	sucrose	-	2,4-D + kin	-	Lo *et al.*, 1980
		shoots	shoots rooted	LS	sucrose	-	-	-	
Cynodon dactylon (Bermuda grass)	SE	young inflorescence	callus formation	N6	sucrose	-	2,4-D	-	Ahn *et al.*, 1985
		callus	plant regeneration	N6	sucrose	-	-	-	
Chloris gayana (Rhodes grass)	SE	mature seeds	callus formation	MS	sucrose	-	2,4-D	yeast extract	Doi *et al.*, 1985
		callus	plant regeneration	MS	sucrose	CH	NAA + BAP	yeast extract	
Cortaderia selloana (Pampas grass)	O	young inflorescence	callus & shoot formation	MS	sucrose	-	2,4-D + BAP	$MgCl_2$	Robacker, 1995
		shoots	shoots rooted	MS	sucrose	-	IBA	-	

Table 2.1 contd.

Species	Route	Explant	Results	Culture medium composition					References
				Nutrients	Sugar	Amino acid	Plant hormones	Other additives	
Dactylis glomerata (Orchard grass)	SE	young leaves	somatic embryo formation	SH	sucrose	-	dicamba	epinephrine[#]	Hanning & Conger, 1986; [#]Kuklin & Conger, 1995
		somatic embryo	plant regeneration	SH	sucrose	-	-	-	
Echinochloa colona (Jungle rice)	SE	leaf base	callus formation	MS	sucrose	-	BA+kin+NAA	-	Samantaray *et al.*, 1997
		callus	plant regeneration	MS	sucrose				
Eragrostis curvula (Weeping lovegrass)	SE	young inflorescence	callus formation	MS	sucrose	-	2,4-D+BA	-	Echenique *et al.*, 1996
		callus	plant regeneration	MS	sucrose				
Lolium multiflorum (Ryegrass)	SE	roots	callus formation	MS	sucrose	-	2,4-D	-	Jackson *et al.*, 1986
		callus	plant regeneration	0.5 MS	sucrose	-	2,4-D +IAA+kin	-	
	O	nodal segment	tiller production (root+shoot)	MS	sucrose	-	BAP	-	Dalton & Dale, 1981; Hussey, 1976
Panicum maximum (Guinea grass)	SE	young leaves	callus formation	MS	sucrose	-	2,4-D	coconut milk	Lu & Vasil, 1981
		callus	plant regeneration	MS	sucrose	-	GA_3	-	
Panicum virgatum (Switchgrass)	O	nodal segment	inflorescence production	MS	maltose	-	BAP	-	Alexandrova *et al.*, 1996a
	SE	caryopse & leaf	callus production	MS	maltose	-	2,4-D + BAP		Denchev & Conger, 1995
		callus	plant regeneration	MS	maltose	-	-	-	
Paspalum notatum (Bahiagrass)	SE	leaf-stem	callus	SH	sucrose	-	Dicamba	-	Shatters *et al.*, 1994
		callus	plant regeneration	SH	sucrose	-	-	-	
Paspalum vaginatum (Seashore paspalum)	SE	immature inflorescence	callus formation	MS mod.	sucrose	-	2,4-D+ BA+NAA	-	Cardona & Duncan, 1997
		callus	plant regeneration	MS	sucrose				

Table 2.1 contd.

Species	Route	Explant	Results	Culture medium composition					References
				Nutrients	Sugar	Amino acid	Plant hormone	Other additives	
*Pennisetum americanum** (Pearl millet)	SE	immature inflorescence	callus production	MS	sucrose	-	2,4-D + kin	$AgNO_3$ + $CoCl_2$ + $NiCl_2$ + cefotaxime + ASA	Pius *et al.*, 1993
		callus	plant regeneration	0.5 MS	sucrose	-	-	as above	
Pennisetum purpureum (Napier grass)	SE	young leaves	callus production	MS	sucrose	-	2,4-D + NAA + BAP	coconut milk	Chandler & Vasil, 1984
		callus	plant regeneration	MS	sucrose	-	GA_3	-	
Poa pratensis (Kentucky bluegrass)	O	nodal segment	plantlet production	0.5 MS	sucrose	gly	-	N & N vitamins	Pieper & Smith, 1988
	SE	mature seeds	callus production	MS	maltose	-	2,4-D + BAP	-	van der Valk *et al.*, 1995
		callus	plant regeneration	MS	maltose	-	-	-	
Saccharum spp (Sugarcane)	SE	leaf roll	callus induction	MS	sucrose	CH	2,4-D	-	Snyman *et al.*, 1996
		callus	plant regeneration	MS	sucrose	CH	-	-	
Setaria anceps (Nandi)	O	nodal segment	shoots formed	MS	sucrose	-	BAP	-	Flachsland *et al.*, 1997
			shoots rooted	MS	sucrose		-		
Tripsacum dactyloides (Eastern gammagrass)	SE	leaf sheath	callus formation	MS	sucrose	-	2,4-D +kin+zea	-	Jeoung *et al.*, 1998
		callus	plant regeneration	MS	sucrose	-	2,4-D +kin+zea	-	
Cereals									
Avena sativa (Oats)	O	immature embryos	callus & shoot formation	B5 & MS	sucrose	-	2,4-D	-	Cummings *et al.*, 1976
		shoots	shoots rooted	B5	sucrose	-	2,4-D	-	
	SE	young leaves	callus production	MS	sucrose	asp	2,4-D	-	Chen *et al.*, 1995
		callus	plant regeneration	N6	sucrose	glu + CH	kin + NAA	-	

Table 2.1 contd.

Species	Route	Explant	Results	Culture medium composition					References
				Nutrients	Sugar	Amino acid	Plant hormone	Other additives	
Hordeum vulgare (Barley)	O	apical meristem	callus production	MS	sucrose	-	IAA+2,4-D+2iP	-	Cheng & Smith, 1975
		callus	shoot production	MS	sucrose	-	-	-	
	SE	immature embryos	callus production	MS	sucrose	-	2,4-D	$CuSO_4$	Dahleen, 1995
		callus	plant regeneration	MS	sucrose	-	-	$CuSO_4$	
Oryza sativa (Rice)	SE	mature seeds	callus production	N6	maltose + lactose	pro + CH	2,4-D	-	Asano & Suguira, 1990; Asano *et al.*, 1994
		callus	plant regeneration	N6	sucrose	-	NAA+ BAP	-	
Setaria italica (Foxtail millet)	SE	shoot apices	callus production	MS	sucrose	-	2,4-D	-	Osuna-Avila *et al.*, 1995
		callus	plant regeneration	MS	sucrose	CH	2,4-D + kin	-	
Sorghum bicolor (Sorghum)	SE	immature embryos	callus production	MS	sucrose	asp + pro	2,4-D	-	Elkonin *et al.*, 1995
		callus	plant regeneration	MS	sucrose	-	IAA + kin	-	
Triticum aestivum (Wheat)	SE	young leaves	callus production	MS	sucrose	-	2,4-D	-	Wernicke & Milkovits, 1984
		callus	plant regeneration	MS	sucrose	-	-	-	
Zea mays (Maize)	SE	immature embryos	callus production	N6	sorbitol	pro + CH	dicamba	NaFeEDTA + KNO_3	Duncan *et al.*, 1985; Swedlund & Locy, 1993
		callus	plant regeneration	N6	sorbitol	-	dicamba + kin	-	
	O	nodal segment	axillary bud multiplication	MS	sucrose	gly	IBA + kin	AS + $NaPO_4$	Raman *et al.*, 1980; Greyson & Walden, 1994
		axillary shoot	root induction	MS	sucrose	gly	IBA + kin	AS + $NaPO_4$	

SE = somatic embryogenesis; O = organogenesis; MS = Murashige & Skoog (1962); N6 = Chu *et al.* (1975); LS = Linsmaier & Skoog (1965); B5 = Gamborg *et al.* (1968); SH = Schenk & Hildebrandt (1972); N & N = Nitsch & Nitsch (1969); 2,4-D = 2,4- dichlorophenoxyacetic acid; NAA = naphthylacetic acid; BAP = benzyl amino purine; GA_3 = gibberellic acid; Dicamba = 3,6-dichloro-o-anisic acid; IBA = 3-indole-butyric acid; IAA = indole-3-acetic acid; kin = kinetin; zea = zeatin; 2iP = 2-isopentyladenine; TDZ = thidiazuron; CH = casein hydrolysate; pro = proline; thio = thioproline; gly = glycine; asp = asparagine; glu = glutamine; pur = purine; ASA = salicyclic acid. AS = adenine sulphate. **Pennisetum americanum* has been renamed *Pennisetum glaucaum* (Wang *et al.*, 2001).

"Somatic embryogenesis is defined as the process in which a bipolar structure resembling a zygotic embryo develops from a nonzygotic cell without vascular connection with the original tissue" (von Arnold *et al.*, 2002). Since the embryos result from somatic cells without the fusion of gametes, they are also referred to as nonzygotic embryos (Gray, 2000). This morphogenic process occurs through a series of stages characteristic of zygotic embryogenesis (Dodeman *et al.*, 1997; Gray, 2000; von Arnold *et al.*, 2002). Somatic embryos can differentiate either directly from the explant without an intervening callus phase or indirectly after a callus phase (Williams and Maheswaran, 1986). Plant regeneration via somatic embryogenesis is achieved through initiation and maintenance of embryogenic cultures, somatic embryo maturation followed by germination and plantlet development.

The developmental programme for embryogenesis is contained within and controlled by the cell (Gray, 2000). Acquisition of embryogenic competence largely relies on de-differentiation, a process whereby existing transcriptional and translation profiles are erased or altered in order to allow cells to set a new developmental programme (Fehér, *et al.*, 2003). The activation of cell division is required to maintain the de-differentiated cell state. Actively dividing de-differentiated cells are referred to as callus, of which there are two types, embryogenic and non-embryogenic. The typical grass embryogenic callus has several well defined characteristics (Vasil, 1987; Gray, 2000). It is compact, highly organised, slow growing and pale white to yellow in colour. It is comprised mainly of tightly packed cells that are small, isodiametric in shape, densely cytoplasmic, highly basophilic, uninucleate, and thin-walled with many small vacuoles. In contrast, non-embryogenic cells are large with no consistent shape, thick-walled, contain large vacuoles and are multi- or e-nucleated (Vasil, 1987; Emons, 1994; Gray, 2000). Only the embryogenic cell has the potential to divide to form a somatic embryo. This occurs through an organised sequence of cell division, enlargement and differentiation. The zygotic and nonzygotic embryos share the same gross pattern of development, with other monocotyledonous species passing through globular, scutellar and coleoptile stages (Gray, 2000).

Formation of somatic embryos in the Poaceae was first demonstrated in the early 1980s (Vasil, 1987), and since then somatic embryogenesis has been described in almost all the species of grasses that have been regenerated in culture (Wang *et al.*, 2001). This

morphogenic regeneration pathway is now considered to be the predominant mode for *in vitro* grass regeneration (Vasil, 1987; Krishnaraj and Vasil, 1995; Wang *et al.*, 2001). Somatic embryogenesis protocols have been established for a number of grasses and a some of those studies are referenced in Table 2.1. In *C. dactylon, in vitro* regeneration via somatic embryogenesis was first reported by Ahn *et al.* (1985, 1987). Other attempts were made by Artunduaga *et al.* (1988, 1989) and more recently by Chaudhury and Qu (2000) and Li and Qu (2002). All of those published accounts used the same explant source, the immature inflorescence. Ahn *et al.* (1985) found that alternative explants (nodes, root tips, young leaves, mature caryopses) formed callus but with no plant regeneration. To date there does not appear to be any published accounts, apart from the present study, on the *in vitro* regeneration of *C. dactylon* from young leaf bases.

The development of new technologies has facilitated an improved understanding of the physiological and developmental state of explants. This has resulted in further published accounts on the optimization of somatic embryogenesis protocols for members of the Poaceae (Krishnaraj and Vasil, 1995; Wang *et al.*, 2001). Also, factors that influence somatic embryogenesis also impact upon other types of *in vitro* regeneration; one of the most important is choice of explant. Immature plant organs that still maintain meristematic activity and competence (such as immature embryos, young inflorescences and bases of young leaves), ensure the establishment of embryogenic grass and cereal cultures (Vasil and Vasil, 1994). Mature leaves and inflorescences produce only non-embryogenic callus or show no *in vitro* response (Vasil, 1987). Thus, it appears that only tissue with cells in a meristematic and undifferentiated state can be stimulated to divide and form callus (Vasil, 1987; Gray, 2000). The nodal segment and young inflorescences are mainly used to initiate organogenic cultures (Table 2.1). Although plants have been regenerated from mature nodal segments (Pieper and Smith, 1988; Flachsland *et al.*, 1997), young explants are more responsive in culture than older explants, especially with regard to indirect organogenesis and the production of shoot-forming callus (Robacker, 1995; Vikrant and Rashid, 2001).

In vitro plant regeneration was achieved by many authors using mature embryos as the explant source (Asano and Suguira, 1990; Denchev and Conger, 1995; van der Valk *et al.*, 1995). Pedrosa and Vasil (1996), however, reported that the embryogenic response of *Zea diploperennis* embryos decreased with increasing age. In that study it appeared

that very young (≤ 1 mm) and mature embryos (≥ 4 mm) were not morphogenically competent and embryos of 1-2 mm in size produced embryogenic callus most frequently. Thus it seems that only embryos at a specific developmental stage are competent to produce embryogenic callus. Recent studies in wheat have confirmed this (Vikrant and Rashid, 2001; Wang *et al.*, 2003).

Although immature embryos and young inflorescences serve as good sources for embryogenic callus in the Poaceae, they are not readily available throughout the year and certain hybrid species do not flower or produce seed. Therefore in those cases, the establishment of regeneration protocols using young leaves is essential. Such regeneration protocols have been established for a variety of grasses (Lu and Vasil, 1981; Kuklin and Conger, 1995; Samantaray *et al.*, 1997) and cereals (Wernicke and Milkovits, 1984; Chen *et al.*, 1995). Only the basal end of the young leaves can be stimulated to produce embryogenic callus (Rajasekaran *et al.*, 1987a; Chen *et al.*, 1995; Samantaray *et al.*, 1997). It appears that a developmental gradient is present in young leaves and responsiveness to produce embryogenic callus decreases with increasing distance from the base. This developmental gradient seems to be linked to varying levels of endogenous hormones. The leaf base has been found to contain higher levels of IAA and BA than the distal regions (Rajasekaran *et al.*, 1987b; Wenck *et al.*, 1988). This relationship between endogenous auxin content of young leaves and its influence on embryogenesis does not appear to have been further investigated in both the grasses and cereals. Possibly since in most subsequent studies if one explant type failed to produce embryogenic callus then an alternative explant was selected or the culture conditions were modified.

Evidence in the literature suggests that explant type and suitable culture conditions can influence the morphogenic route of development. For example, Pedrosa and Vasil (1996) reported that embryogenic callus was initiated from immature embryos (*Zea diploperennis*), whereas young leaves and immature inflorescences yielded only non-embryogenic callus. Mature and immature *Triticale* spp embryos produced embryogenic callus and plants were regenerated via somatic embryogenesis, while the leaf base produced organogenic callus (Vikrant and Rashid, 2001). It was also reported that in tall wheatgrass (*Thinopyrum ponticum*), the immature inflorescence produced more

embryogenic callus than the immature embryo, mature embryo and caryopsis (Wang *et al.*, 2003).

In addition to explant type, it seems that the efficiency of *in vitro* regeneration is also dependent upon genotype. Early reports for wheat (Maddock *et al.*, 1983), maize (Tomes and Smith, 1985) and barley (Walmsley *et al.*, 1995) showed that genotypes of the same species have different *in vitro* responses, even with the same explant type. Those differences could be due to variations between genotypes and their susceptibility to genetic programming and re-programming of embryogenically competent cells by external factors (Joy and Thorpe, 1999; Fehér *et al.*, 2003). Therefore, in many cases the conditions for plantlet regeneration were successfully modified for each genotype (Saidi *et al.*, 1997; Cardona and Duncan, 1997; Bregitzer *et al.*, 1998; Bai and Qu, 2000; Nuutila *et al.*, 2002; Przetakiewicz *et al.*, 2003). In some cases, recalcitrant (usually unresponsive to *in vitro* culture) genotypes have been stimulated to respond by changing the explant and culture conditions. For example mature barley embryos produced three to eight fold more shoots than organogenic callus derived from the scutella, leaf base or apical meristem (Ganeshan *et al.*, 2003).

The MS (Murashige and Skoog, 1962) macronutrient formulation is reported for 25% of all published *in vitro* work since it became common practice to compare this medium or dilutions thereof when developing regeneration protocols for a particular species (Leifert *et al.*, 1995). The same is true for many grasses and cereals, with the MS nutrient medium (both macro- and micronutrients) supporting plant propagation (Krishnaraj and Vasil, 1995; Wang *et al.*, 2001). The MS medium is used to initiate both organogenic and embryogenic callus and also supports axillary bud development and rooting. According to Leifert *et al.* (1995), the frequency of use of the MS formulation for inducing embryogenic and organogenic callus cultures (in the Poaceae) was 70% and 62%, respectively. Shoots were rooted on either full- or half-strength MS medium. The half-strength MS nutrient medium was also found to improve plant regeneration (Pius *et al.*, 1993).

A review of published studies has shown that for certain grasses and cereals nutrient formulations, other than MS, proved to be successful for plant regeneration (Chen *et al.*, 1977; Lo *et al.*, 1980; Ahn *et al.*, 1985; Hanning and Conger, 1986; Shatters *et al.*,

1994). For example, the Linsmaier and Skoog (1965) nutrient formulation was used to initiate organogenic callus in *Agropyron* spp. (Lo *et al.*, 1980) and the N6 medium supported plant regeneration in *Oryza sativa* (Asano *et al.*, 1994) and *Avena sativa* (Chen *et al.*, 1995). That nutrient formulation was also reported to improve callus production in *C. dactylon* (Ahn *et al.*, 1985) but other workers preferred the MS formulation (Artunduaga *et al.*, 1988; Chaudhury and Qu, 2000). The SH (Schenk and Hildebrandt, 1972) medium was most suitable for the initiation of embryogenic callus from *Dactylis glomerata* (Hanning and Conger, 1986) and *Paspalum notatum* (Shatters *et al.*, 1994) explants, while the B5 nutrients were used in an early study to initiate organogenic callus from *Avena sativa* (Cummings *et al.*, 1976).

A carbon source is essential for *in vitro* regeneration since it provides for the energy demands of cells in culture (George, 1996). To date sucrose (20-30 g l^{-1}) has served as the primary carbon source in nearly all studies involving the culture of grasses and cereals (Table 2.1). Vasil and Vasil (1994) reported that increased sucrose concentration (20-120 g l^{-1}) resulted in increased callus production and plant regeneration in some grass species. Although Pedrosa and Vasil (1996) found that increased sucrose concentration improved callus production, the resulting *Zea diploperennis* somatic embryos did not germinate. Reports in the literature seem to suggest that a combination of species type and the appropriate carbon source influences callus production. For example, the substitution of sucrose with maltose resulted in enhanced embryogenesis in *Panicum virgatum* (Denchev and Conger, 1995), *Poa pratensis* (van der Valk *et al.*, 1995), *Triticale* spp (Ainsley and Aryan, 1998) and *Avena sativa* (Nuutila *et al.*, 2002). Maltose was also reported to enhance organogenic callus production in mature barley embryos (Ganeshan *et al.*, 2003). In other cultures sorbitol (Swedlund and Locy, 1993) and a combination of sucrose and sorbitol (Okamoto *et al.*, 1996) were successfully used.

Plant hormones are the most likely candidates as agents responsible for regulating the developmental switches in cells, with auxins and cytokinins inducing cell division and differentiation (Fehér *et al.*, 2003). The induction of somatic embryogenesis in grasses is reliant upon a strong auxin, usually 2,4-D (Krishnaraj and Vasil, 1995; Wang *et al.*, 2001). This auxin is often the only growth regulator required to trigger embryogenesis in many grass species, however, in some cultures 2,4-D is supplemented with a strong

cytokinin (Denchev and Conger, 1995; Echenique *et al.*, 1996; Fei *et al.*, 2002). In other cases alternative auxin sources proved to be more efficient at inducing embryogenic callus than 2,4-D. Pedrosa and Vasil (1996) reported that picloram is preferred for the culture of *Zea diploperennis*, whilst others found that dicamba is most suited for *Triticale* spp (Ainsley and Aryan, 1998) and *Paspalum notatum* (Grando *et al.*, 2002).

It is not known what triggers cells of certain species to follow the organogenic rather than the somatic embryogenesis morphogenic route of development. It has been suggested that a combination of plant growth regulators and explant type influences the production of either shoot-forming or embryogenic callus. It was reported for *Eragrostis curvula* (Echenique *et al.*, 1996) and *Buchloe dactyloides* (Fei *et al.*, 2002) that a low concentration of 2,4-D and BA induced organogenic callus from the immature inflorescence whilst an increased concentration of both these plant hormones resulted in embryogenic callus. In another study, culturing the leaf base (*Triticale* spp) on a medium with a low 2,4-D concentration induced shoot-forming callus whereas an increased level of this auxin stimulated the mature embryos of this species to produce embryogenic callus (Vikrant and Rashid, 2001).

Direct organogenesis in the grasses, axillary bud development and shoot multiplication, usually result from the addition of a strong cytokinin (e.g. BAP, kinetin) to the culture medium (Alexandrova *et al.*, 1996a; Flachsland *et al.*, 1997). Shoots are usually rooted in hormone-free media or media supplemented with a low level of auxin (Lo *et al.*, 1980; Jullien and Tran Thanh Van, 1994; Flachsland *et al.*, 1997). Flachsland *et al.* (1997) found that a hormone-free medium with a high sucrose concentration (80 g l^{-1}) improved the rooting of *Setaria anceps* shoots. Similarly Cardona and Duncan (1997) reported that an increased sucrose content (80 g l^{-1}) of the culture medium, when compared to the callus induction medium, stimulated root development of the newly germinated somatic embryos (*Paspalum vaginatum*).

Although it has been suggested that complete embryo maturation is not necessary in order to obtain plants from somatic embryos, it is essential for high plant recovery (Gray, 2000). A hormone-free medium (Shatters *et al.*, 1994; Dahleen, 1995; Samantaray *et al.*, 1997) or media with low levels of plant growth regulators (Kebebew *et al.*, 1998; Bai and Qu, 2000; Nuutila *et al.*, 2002; Wang *et al.*, 2003) are traditionally

known to stimulate somatic embryo maturation and germination in the grasses. In some gymnosperms alternative approaches to somatic embryo maturation have been investigated since traditional ones failed to stimulate germination (Capuana and Debergh, 1997; Norgaard, 1997). Those alternative approaches are based on the theory that maturation drying is an integral part of the development of most seeds (zygotic embryos) and subsequent hydration usually leads to germination (Bewley and Black, 1985). In fact many seeds will not germinate unless they are subjected to a drying phase. Dehydration can be achieved by culturing somatic embryos in a desiccator or by exposing them to a laminar air-flow. Although not a common approach for the grasses, *Dactylis glomerata* (Gray, 1987) and *Zea mays* (Compton *et al.*, 1992) somatic embryos were dehydrated by physical means, resulting in successful somatic embryo germination for both species.

In this study the objective was to establish both organogenesis and somatic embryogenesis regeneration systems for *C. dactylon*, using vegetative explant material that was common to all *C. dactylon* genotypes and available throughout the year. Both regeneration routes were pursued in order to ensure the establishment of an efficient *in vitro* propagation system for *C. dactylon*. These *in vitro* regeneration systems would provide an alternative means for the vegetative propagation of *C. dactylon* and the indirect somatic embryogenesis approach would be used to initiate the required meristematic callus cells.

2.2 Materials and Methods

2.2.1 Plant material and Growth conditions

Plant material for the establishment of *in vitro* regeneration protocols were obtained from *Cynodon dactylon* parent plants grown from seed supplied by the Vegetation Unit of the Chamber of Mines (South Africa). Explant material was not harvested from *C. dactylon* genotypes collected from the acidic mine tailings since this genetic material was potentially valuable. Plants were grown from seed in soil in plastic pots (3000 cm^3) and maintained in the greenhouse (25°C day/18 °C night). A single parent plant was selected and propagated in the greenhouse using macrocuttings, each comprising a node with axillary buds and root primordia. These cuttings grew into mature plants after two months. These plants were grown in soil and watered daily. Fertiliser (N: P: K; 2: 3: 2; 6.3% N, w/v) was applied (3.3 g kg^{-1}) once every two months. To counteract aphids,

during the summer months, plants were sprayed once every six weeks with a solution of 1 ml l^{-1} Metasystox R (Bayer AG, Germany). Plants collected from mine tailings were grown in soil in plastic pots and kept in the greenhouse.

2.2.2 Organogenesis studies

Stolon nodal segments, represented by a node with axillary buds and 10 mm of the internode above and below the node, were excised and the nodal sheath removed to expose the buds. They were sterilised for 20 minutes in 1% (v/v) sodium hypochlorite that contained a few drops of Tween 20. Explants were cultured on MS salts and vitamins (Murashige and Skoog, 1962) and 30 g l^{-1} sucrose for 10 days. Alternatively, explants were placed on shoot multiplication media consisting of MS salts, White (1943) vitamins, 30 g l^{-1} sucrose, 0.22 g l^{-1} $CaCl_2$, 0.014 g l^{-1} $FeSO_4$, 1 mg l^{-1} citric acid, 1 mg l^{-1} ascorbic acid and combinations of kinetin (6-furfurylaminopurine) (1.5, 3 and 5 mg l^{-1}) and IBA (indole butyric acid) (1 and 3 mg l^{-1}) for 10 days. The shoots were rooted on MS salts and vitamins, 30 g l^{-1} sucrose, and 2 mg l^{-1} IBA. All media contained 10 g l^{-1} agar and the pH was set at 5.6 - 5.8, prior to autoclaving. Other culture conditions were 1 explant/10 ml of medium in a 20 x 90 mm glass tube which was maintained in the growth room. All cultures were grown under a photoperiod of 16 h light at a photosynthetic photon flux density of 37 μmol. m^{-2} s^{-1} and a temperature of 25 °C day/20 °C night.

2.2.3 Somatic embryogenesis studies

Explant preparation

Tillers were harvested from the mature parent plants (initially grown from seed and then bulked-up using vegetative means) and kept moist in a container filled with tap water until required. The leaf sheath was unfurled and the outer leaves peeled away to obtain the three youngest leaves. During sample preparation the explants were temporarily stored in half strength MS and 1 g l^{-1} sucrose. Whole leaves were sterilised for 15 minutes in 1% (v/v) sodium hypochlorite that contained a few drops of Tween 20. They were then cut into four regions as shown in Figure 2.1. In all subsequent studies, region D (10 mm) of the youngest leaf served as the explant.

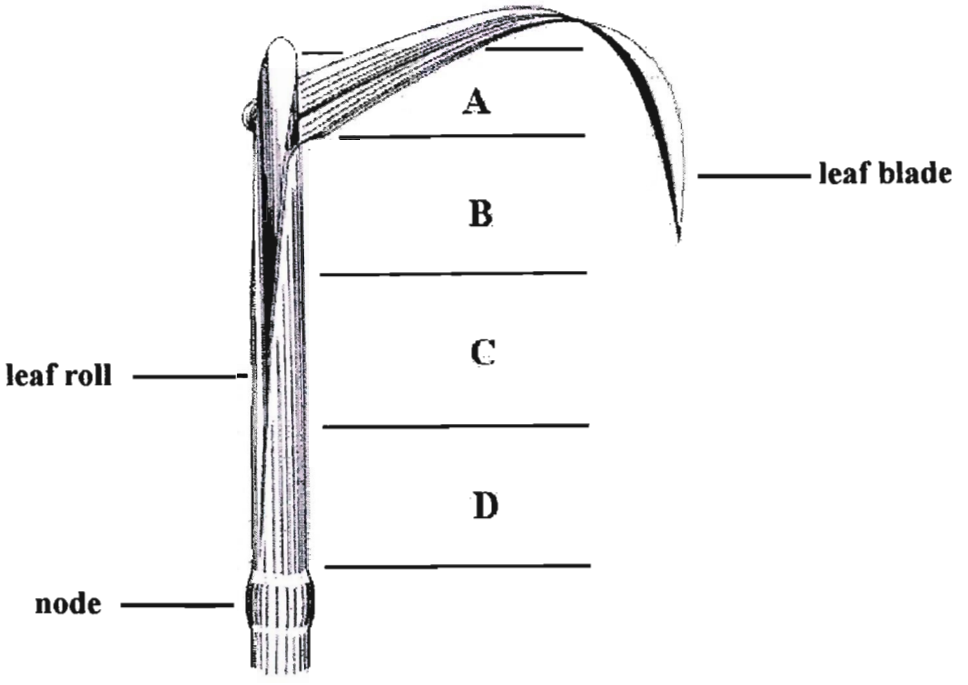

Figure 2.1 A schematic representation of a grass leaf depicting the various regions (A to D) that were evaluated for callus formation (van Oudtshoorn, 1992).

Callus induction

Several basal nutrient formulations (Murashige and Skoog 1962; Gamborg *et al.* 1968; Schenk and Hildebrandt 1972; Chu *et al.* 1975) were initially tested. Thereafter, the Murashige and Skoog (1962) nutrient medium was used to select the most appropriate carbon source, *viz.* 30 and 60 g l^{-1} sucrose, 15 g l^{-1} each of sucrose and sorbitol, 30 g l^{-1} sorbitol, and a combination of 23 g l^{-1} sucrose and 1 g l^{-1} each of ribose, xylose, arabinose, glucose, mannose, galactose and fructose. The most effective auxin type and concentration were selected in a similar manner, on MS nutrient medium. Auxins tested were 2,4-D (2,4-dichlorophenoxyacetic acid 1, 3 and 5 mg l^{-1}); 3 mg l^{-1} picloram (4-amino-3,5,6-trichloropicolinic acid) and 1.5 mg l^{-1} 2,4-D and 1.5 mg l^{-1} picloram. All media contained 10 g l^{-1} agar and the pH was between 5.6 and 5.8, prior to autoclaving. The explants were placed on callus induction media (six explants/30 ml in a 95 mm Petri dish), in the dark at 25 °C for 6 weeks, with one subculture onto fresh nutrient medium after 2 weeks.

Plant regeneration

Compact embryogenic callus regions were selected visually and transferred onto several regeneration media. These all contained MS, 30 g l^{-1} sucrose, 10 g l^{-1} agar and various additives, viz. 10 g l^{-1} activated charcoal, 12 mg l^{-1} ABA (abscisic acid) and 40 g l^{-1} PEG 6000 (polyethyleneglycol), pH 5.6 - 5.8. Some calli were subjected to a physical drying treatment, that involved leaving the uncovered Petri dishes containing embryogenic calli on culture media, under a laminar flow stream (250 Pa) for 1, 3 or 6 h. Thereafter, the calli were cultured on MS, 30 g l^{-1} sucrose and 10 g l^{-1} agar. Media were dispensed into 95 mm Petri dishes and cultures (4 calli pieces/dish) were maintained in the growth room (16 h light/8 h dark photoperiod, 25 °C day/20 °C night and 200 μE m^{-2} s^{-1}). The treatments were evaluated by recording the number of calli that contained germinating embryos and by counting the germinating embryos in single callus clumps of known fresh mass, using a Wildt dissecting stereomicroscope.

Hardening-off

Plantlets regenerated through both organogenesis and somatic embryogenesis were acclimatised to greenhouse conditions. The gelatinous nutrient medium was washed from the roots and the plantlets were potted into moist soil in plastic pots (200 cm^3), enclosed in polythene bags, and placed in the same greenhouse as parent plants (25 °C day/18 °C night). The bags were opened daily for increasingly longer periods for one week, after which they were removed permanently.

Plants produced via nodal cuttings were harvested after 10 days, 1 month or 3 months of growth in the greenhouse. The relative growth rates [$(Mass_1 - Mass_0) / Mass_0$ = g g^{-1} $month^{-1}$] of *in vitro*-produced and macropropagated plants were calculated using the dry mass of each plant type.

2.2.4 Photography

Illustrative stages of somatic embryo development were recorded using a Wildt Photoautomat MPS 55 system. A high contrast black and white film (Tech Pan ASA 125) was used to photograph the embryos on a phase contrast stage. The rooted plantlet was captured using a Nikon FM2 camera with a 60 mm Mikro Nikkor macro lens.

2.2.5 Statistical analyses

The Statgraphics Plus Statistical Graphics System Version 7.0, computer software produced by Manugistics Inc. and Statistical Graphic Corporation, was used for all analyses. The data were initially tested for normality using the Kolmogorov-Smirnoff test ($p > 0.05$). Data were then analysed using a One-Way Analysis of Variance (ANOVA) ($p < 0.05$). If more than two variables were compared then a Scheffé's multiple range test was performed ($p < 0.05$). Statistical significance was denoted between variables by dissimilar lower case alphabet characters.

2.3 Results

2.3.1 Organogenesis

Influence of growth regulators on plantlet production

Shoots and roots developed almost simultaneously from *C. dactylon* nodal segments (Figure 2.2) within 10 days of culture initiation on hormone-free MS nutrient medium, but the yield was very low (1 plant/explant) (Table 2.2). Although all explants produced shoots, only 36% of nodes produced multiple shoots, and of these rooting efficiency was 77%. In an attempt to increase shoot production, nodes were cultured on IBA and/or KIN containing-media for 10 days (Table 2.2). However, as these shoots did not root on the multiplication media, it was necessary to transfer them onto a rooting medium for a further 10 days. The results indicated that the addition of 5 mg l^{-1} KIN had a significant positive effect on shoot yield but reduced subsequent rooting and, consequently, plant yield (Table 2.2).

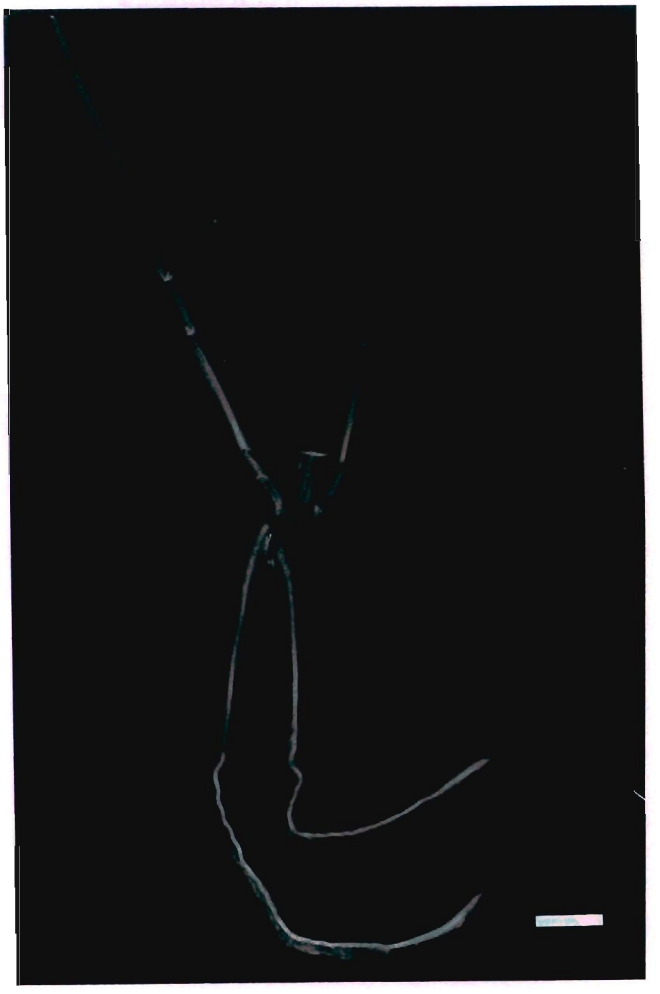

Figure 2.2 Plantlet regenerated from nodal segment via organogenesis. Shoot and root development were achieved in 10 days on hormone free MS nutrient media. Scale bar = 6 mm.

Table 2.2 The effect of plant growth regulators on shoot and root proliferation from nodal explants

Plant hormone ($mg\ l^{-1}$)	% explants with shoots	% explants with > 1 shoot	% explants rooted	Plant yield/explant
none*	100 b	36 b	77 c	1.0
1.5 kin + 3 IBA	56 a	0 a	0 a	0
3 kin + 1 IBA	100 b	55 bc	46 b	0.7
5 kin	100 b	64 c	36 b	0.6

The media contained MS, White (1943) vitamins, 0.22 $g\ l^{-1}$ $CaCl_2$, 0.014 $g\ l^{-1}$ $FeSO_4$, 1 $mg\ l^{-1}$ citric acid, 1 $mg\ l^{-1}$ ascorbic acid, 30 $g\ l^{-1}$ sucrose and 10 $g\ l^{-1}$ agar. The rooting medium included MS, 30 $g\ l^{-1}$ sucrose, 10 $g\ l^{-1}$ agar and 2 $mg\ l^{-1}$ IBA. * = in this hormone-free MS nutrient treatment, shoot and root proliferation were achieved in a single medium. Data were recorded 10 days after culture initiation for shoot proliferation and 20 days later for root induction. Mean values for the hormone treatments represented by different alphabetical letters were significantly different (Scheffé's multiple range test $n = 20$, $p < 0.05$).

Yields of in vitro- and conventionally-produced plants

Plants with both shoots and roots were produced in 10 days via the organogenesis method. The *ex vitro* growth of these plants was compared with those of traditionally-grown plants (i.e. propagated via cuttings in soil not *in vitro*). After ten days, the *in vitro*-produced plantlets were transferred to the greenhouse, their dry mass was determined and compared with that of conventionally-produced plants (Table 2.3). Dry mass determinations were then repeated one and three months later. The results showed that, after one month in the greenhouse, there was no significant difference in dry mass between the two types of plants. However, the *in vitro*-produced plantlets had a higher relative growth rate (182.2 $g\ g^{-1}\ month^{-1}$) than traditionally grown plants (36.8 $g\ g^{-1}\ month^{-1}$).

Table 2.3 A comparative study on the performance of plants regenerated from nodal segments *in vitro* and from cuttings (nodal segments)

Propagatory route	10 days mass (g)	1 month mass (g)	1 month RGR ($g\ g^{-1}\ month^{-1}$)	3 months mass (g)	3 months RGR ($g\ g^{-1}\ month^{-1}$)
in vitro	0.01 a	2.07 a	182.2 b	98.87 a	15.6 a
cuttings	0.053 b	2.03 a	36.77 a	108.91 a	17.6 a

Total dry mass was recorded after 10 days, 1 month and 3 months and relative growth rates (RGR) were calculated after 1 and 3 months. Data were recorded 10 days after culture initiation. Dissimilar alphabet characters represent a statistical significance using a Scheffé's multiple range test ($n = 10$, $p < 0.05$).

The *in vitro*-produced and traditionally-grown plants had similar relative growth rates after three months (Table 2.3). Therefore, even though *in vitro*-produced plants were initially (after 10 days of *in vitro* growth) five times smaller than those traditionally grown, this did not negatively affect their subsequent growth in the *ex vitro* environment.

2.3.2 Somatic embryogenesis

The morphogenic process of somatic embryogenesis in C. dactylon

When basal segments from young *C. dactylon* leaves were cultured on MS nutrient media supplemented with 3 mg l^{-1} 2,4-D, callus proliferation was observed after one week incubation in the dark. *Cynodon dactylon* callus follows the well defined characteristics that have been defined for other grasses (Vasil, 1987; Gray, 2000). Embryogenic (compact, opaque and white to pale yellow, with small dense cells each showing a single prominent nucleus) (Figure 2.3A) and non-embryogenic calli (soft, watery and gelatinous, with large, highly vacuolated cells) (Figure 2.3B) were distinguishable only after four weeks. Six weeks after culture initiation, fully-formed globular, notched, embryos (Figure 2.4A, B) were visible on the surface of the compact callus. At this time the embryogenic calli (selected visually and separated from the non-embryogenic regions) were transferred onto regeneration media. One week later, shoot primordia and roots (Figure 2.4C) were visible and seedling establishment occurred in the following six to eight weeks (Figure 2.4D).

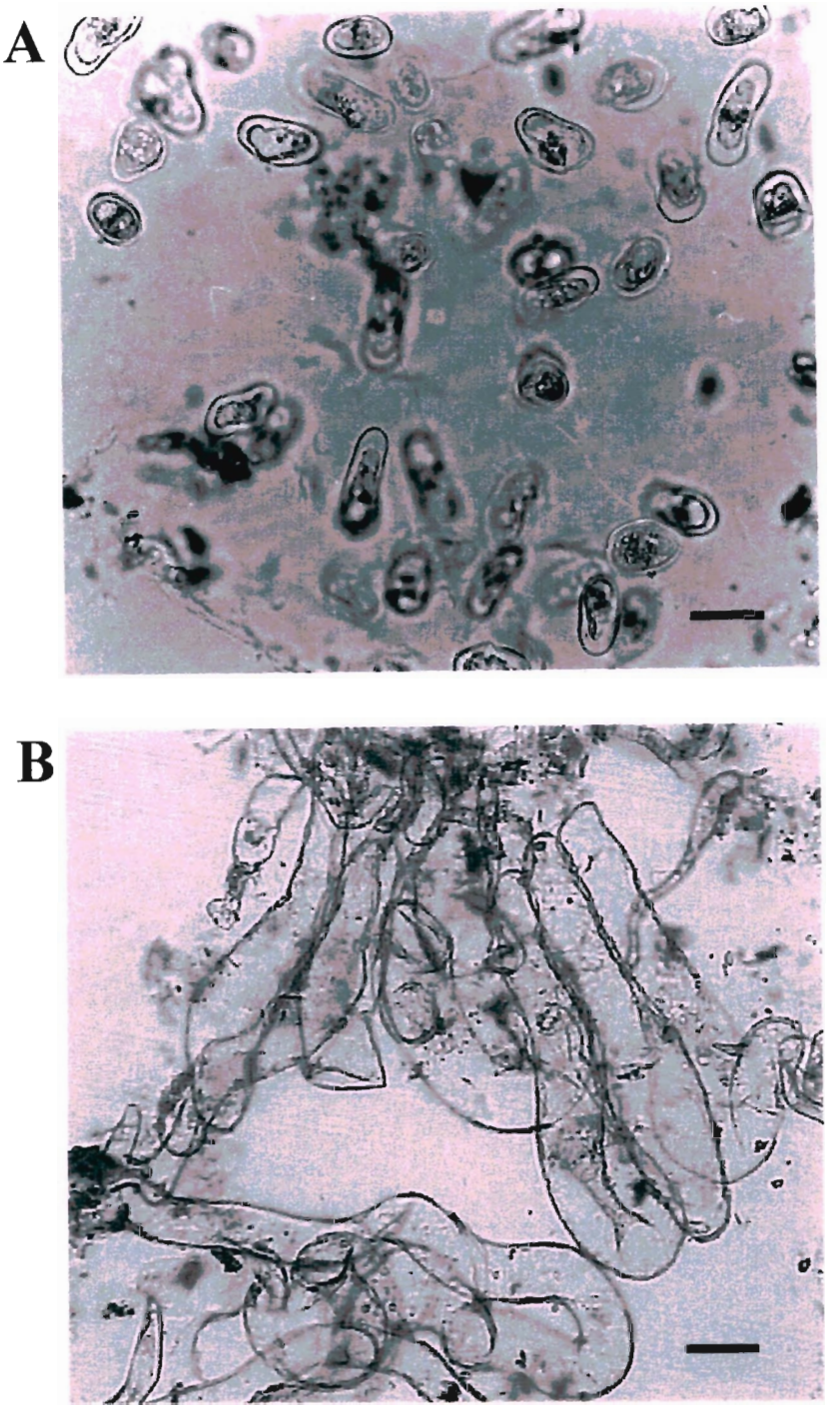

Figure 2.3 Two types of cells that characterise *C. dactylon* callus. Embryogenic cells (A) that are small with prominent nuclei and large tubular shaped non-embryogenic cells (B). Scale bar = 0.2 mm.

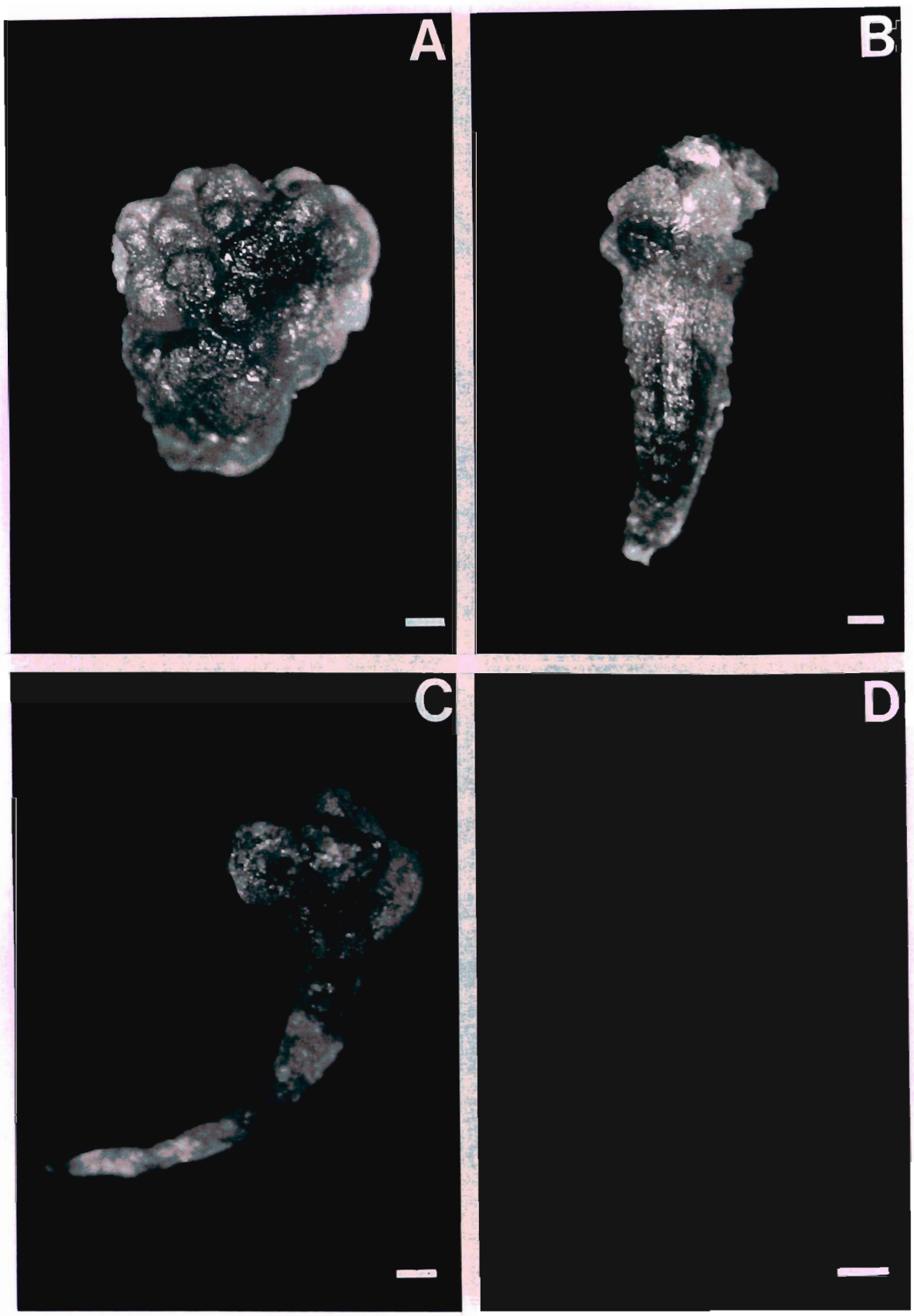

Figure 2.4 Somatic embryo development in *C. dactylon*. The stages shown are early globular notched (A), late globular notched (B) and mature (C). The mature embryos were stimulated to germinate after a 3 h fast-drying treatment and developed into plantlets (D) on MS nutrients supplemented with 30 g l^{-1} sucrose and 10 g l^{-1} agar. Scale bar for A-C = 0.2 mm and scale bar for D = 6 mm.

Factors influencing the production of embryogenic callus

Different parameters were investigated to optimise the induction of embryogenic callus from *C. dactylon* leaf material. These included the source and age of the leaf explant, the nutrient formulation, type and concentration of sugar and auxin in the culture medium. In this study, the three most immature leaves in a leaf roll were initially used and referred to as leaf 1 (youngest), leaf 2 and leaf 3 (oldest). Each leaf was divided into four regions, as shown in Table 2.4, and cultured on MS nutrient medium containing 3 mg l^{-1} 2,4-D. Callus production was directly related to leaf age and region, with 80% of explants from the basal end of the youngest leaf (region D of leaf 1) producing callus (Table 2.4). Observations made, using the dissecting microscope, indicated that this callus was highly embryogenic. The basal segment of young leaves was used in all subsequent investigations.

Table 2.4 The effect of leaf age and region on callus induction

Leaf region	% explants with callus		
	Leaf 1	Leaf 2	Leaf 3
A	0 a	0 a	0 a
B	5 a	57 c	11 b
C	60 b	0 a	0 a
D	80 c	14 b	0 a

Leaves were cultured on MS nutrient medium containing 30 g l^{-1} sucrose, 10 g l^{-1} agar and 3 mg l^{-1} 2,4-D. Leaf 1 = youngest leaf, leaf 3 = older outer leaf. Each leaf was divided into four regions (A, B, C, and D). Data were recorded 28 days after culture initiation. Dissimilar alphabet characters represent a statistical significance using a Scheffé's multiple range test ($n = 20$, $p < 0.05$).

In *C. dactylon*, the MS nutrient formulation (Table 2.5) and 30 g l^{-1} sucrose (Table 2.6) were the most effective in the production of embryogenic callus (80% explants producing callus). Although the MS nutrient formulation is the most commonly used in grass propagation, other less frequently used nutrient formulations were also tested (SH, N6 and B5). These were found to be much less effective with regard to embryogenic callus induction than MS (Table 2.5). An increase in sucrose concentration was found to have a marked negative effect on callus induction. A doubling in the concentration of

sucrose from 30 to 60 g l^{-1} resulted in a 35% decrease in callus production (Table 2.6). None of the explants cultured on media with sorbitol produced callus but 50% of those on media containing a combination of sucrose and sorbitol did. A combination of sucrose with seven simple sugars (ribose, xylose, arabinose, glucose, mannose, galactose and fructose) also resulted in low callus induction (Table 2.6).

Table 2.5 The influence of nutrient formulations on callus induction from basal leaf segments

Nutrient formulation	% explants with callus
MS	80 c
SH	36 b
N6	18 a
B5	26.5 b

MS = Murashige and Skoog, 1962; B5 = Gamborg *et al.* 1968; SH = Schenk and Hildebrandt, 1972; N6 = Chu *et al.* 1975. Other media constituents included 30 g l^{-1} sucrose, 10 g l^{-1} agar and 3 mg l^{-1} 2,4-D. Data were recorded 28 days after culture initiation. Dissimilar alphabet characters represent a statistical significance using a Scheffé's multiple range test ($n = 40$, $p < 0.05$).

Both types of auxin (2,4-D and picloram) promoted callus formation at all tested concentrations and combinations (Table 2.7). The auxin 2,4-D proved to be far more effective than picloram, with 3 mg l^{-1} 2,4-D resulting in 80 % of the explants producing callus. It should be noted that a further increase in 2,4-D concentration resulted in decreased callus production.

Table 2.6 The effect of various sugar types and concentrations on callus induction from basal leaf segments

Sugar Type	Sugar $g\ l^{-1}$	% explants with callus
sucrose	30	80 c
sucrose	60	45 b
sorbitol	30	0 a
sucrose + sorbitol	15 + 15	50 b
sucrose + simple sugars*	23 + 7	39 b

The treatments also included MS nutrients, 10 $g\ l^{-1}$ agar and 3 $mg\ l^{-1}$ 2,4-D. * Simple sugars included 1 g each of ribose, xylose, arabinose, glucose, mannose, galactose and fructose. Data were recorded 28 days after culture initiation. Dissimilar alphabet characters represent a statistical significance using a Scheffé's multiple range test ($n = 40$, $p < 0.05$).

Table 2.7 The effect of auxin type and concentration on callus induction from basal leaf segments

Auxin Type	Auxin $mg\ l^{-1}$	% explants with callus
2,4-D	1	13.1 a
	3	80 d
	5	52.9 b
picloram	3	46.2 b
2,4-D + picloram	1.5 + 1.5	68 c

The callus induction medium also contained MS nutrients, 30 $g\ l^{-1}$ sucrose and 10 $g\ l^{-1}$ agar. Data were recorded 28 days after culture initiation. Dissimilar alphabet characters represent a statistical significance using a Scheffé's multiple range test ($n = 40$, $p < 0.05$).

Somatic embryo germination

Embryogenic calli resulting from culture medium that contained MS nutrients, 30 g l^{-1} sucrose, 10 g l^{-1} agar and 3 mg l^{-1} 2,4-D were exposed to different regeneration treatments, none containing 2,4-D (Table 2.8). Only 50% of the somatic embryos transferred to MS nutrient medium, with or without 10 g l^{-1} activated charcoal, germinated. Abscisic acid failed to stimulate germination in any of the cultures. The chemical dehydration approach (40 g l^{-1} PEG) also failed to stimulate embryo germination but a physical dehydration treatment (180 min drying) was much more successful, as all the calli germinated.

Table 2.8 The influence of various treatments on the germination of somatic embryos

Treatment	% calli with germinating embryos	% embryo germination
none	50 b	50 b
10 g l^{-1} activated charcoal	45 b	48 b
12 mg l^{-1} ABA	0 a	0 a
40 g l^{-1} PEG	0 a	0 a
drying 1 h	40 b	63 b
drying 3 h	100 d	100 d
drying 6 h	70 c	80 c

All treatments included MS nutrients, 30 g l^{-1} sucrose and 10 g l^{-1} agar. Clumps of calli that were cultured on regeneration media contained approximately 200 embryos g^{-1} fresh mass. The cultures were incubated in the light (16h) and observations recorded 2 weeks after culture initiation. Data were recorded 28 days after culture initiation. Dissimilar alphabet characters represent a statistical significance using a Scheffé's multiple range test ($n = 20$, $p < 0.05$).

2.4 Discussion

2.4.1 Considering somatic embryogenesis in the grasses

The process of somatic embryogenesis in *C. dactylon* (i.e. callus induction, selection of embryogenic callus, embryo maturation and plant regeneration) is similar to those described for other grasses (Samantaray *et al.*, 1997; Saxena and Dhawan, 1999; Wang *et al.*, 2003). However, in earlier studies the immature inflorescence has been used to

regenerate *C. dactylon* via somatic embryogenesis (Ahn *et al.*, 1987; Artunduaga *et al.* 1988, 1989). Although those authors used a different explant source, the process of somatic embryogenesis described by them is similar to this study. The stages of somatic embryo development outlined for *C. dactylon* (Figure 2.4) appeared to fit the pattern of development for wheat globular embryos (Fischer and Nehaus, 1995; Fischer *et al.* 1997). The shape of the late globular notched (Figure 2.4B) and mature (Figure 2.4C) *C. dactylon* embryos indicate that these structures are bipolar, with developing shoot and root regions. These developing bipolar embryos confirm the occurrence of somatic embryogenesis. Zygotic and non-zygotic embryos share the same gross pattern of development, with both typically passing through globular, scutellar and coleoptilar stages in monocotyledonous plants (Gray, 2000). Generally the anatomy and morphology of well-developed non-zygotic embryos are faithful to its corresponding zygotic counterpart, such that differences and mutations can be identified in its infancy. Identifying the various stages of somatic embryo development is, therefore, important since mutated embryos will fail to germinate (Dodeman *et al.*, 1997; Fischer *et al.*, 1997). Mature *C. dactylon* embryos were germinated on a hormone-free MS nutrient medium and regenerated plants were acclimatised to the *ex vitro* conditions of the greenhouse.

The basal segments of young *C. dactylon* leaves were selected to initiate callus in this study since the apical regions were found to be less responsive to callus formation than the leaf bases (Table 2.4). It seemed that these young *C. dactylon* leaves, like those of other grasses and cereals (Wernicke and Milkovits, 1984; Wenck *et al.*, 1988; Denchev and Conger, 1994; Chen *et al.*, 1995; Gless *et al.*, 1998; Nuutila *et al.*, 2002) contained a gradient of morphogenic competence from the base to the apex. The older leaves, regardless of region, appeared to be less responsive to callus formation than the young leaf (Table 2.4), suggesting that explants in an early developmental stage and largely made up of meristematic and undifferentiated cells (i.e. leaf base) induces more callus than explants with differentiated mature tissue. Further, Chen *et al.* (1995) reported that the frequency of regenerable callus formation obtained from leaf base segments was considerably higher than immature embryos. This improved response of the former explant type has been linked to high levels of endogenous hormones (IAA and ABA). Those hormones have been shown to have a positive influence on embryogenic callus formation (Rajasekaran *et al.*, 1987b; Wenck *et al.*, 1988). Amongst others, the leaf-

base has been used to regenerate *Tripsacum dactyloides* (Jeoung *et al.*, 1998), *Echinochloa colona* (Samantaray *et al.*, 1997) and *Dactylis glomerata* (Hanning and Conger, 1986) via somatic embryogenesis. It appears, therefore, that explant type and its corresponding endogenous auxin content are important factors controlling embryogenic cell fate (Pasternak *et al.*, 2002; Fehér *et al.*, 2003).

The influences of an exogenous auxin source (primarily 2,4-D) on the induction of somatic embryogenesis in the grasses are well documented (Vasil, 1987; Krishnaraj and Vasil, 1995; Wang *et al.*, 2001). In this study, like for many other grasses, 2,4-D was the only plant growth hormone required for embryogenic callus induction (Kebebew *et al.*, 1998; Bai and Qu, 2000; Lauzer *et al.*, 2000; Wang *et al.*, 2003). In contrast, Chaudhury and Qu (2000), using the *C. dactylon* inflorescence reported that a cytokinin (BAP) was also essential for the induction of embryogenic callus. Although they improved the callus induction medium proposed for *C. dactylon* by other authors (Ahn *et al.*, 1987; Artunduaga *et al.*, 1988), only 20% of explants produced regenerable callus. In contrast callus was initiated from 80% of leaf bases in this study, suggesting that perhaps both choice of explant and genotype influence production of embryogenic callus.

Embryogenic callus in this study was initiated from leaf bases that were cultured on MS nutrient media supplemented with sucrose, 2,4-D and a gelling agent (agar). Murashige and Skoog (1962) based media, supplemented with sucrose, are commonly used in grass tissue culture (Krishnaraj and Vasil, 1995; Wang *et al.*, 2001). As shown in the published literature, alternative nutrient formulations have been successfully used in other work. Of the four nutrient formulations tested in the present study MS proved to be most effective for callus formation (Table 2.5). In an earlier study, embryogenic callus was induced from the *C. dactylon* inflorescence cultured on the N6 nutrient formulation (Ahn *et al.*, 1987). In that work 50% of the inflorescences, cultured on N6 media supplemented with 2,4-D, produced embryogenic callus. In the present study using an alternative explant source on the N6 medium that was supplied with 2,4-D, 20% of the leaf bases produced callus. Thus it appears that, like with many other grasses, the nutrient formulation in combination with explant type, genotype and auxin source influences embryogenic callus induction in *C. dactylon*.

Nuutila *et al.* (2002) reported that the interaction between both the sugar and auxin influences embryogenesis in barley. They showed that a combination of maltose and 2,4-D, rather than sucrose and 2,4-D, enhanced embryogenic callus production in that cereal. In this study the auxin 2,4-D was tested in conjunction with sucrose, sorbitol and a combination of sucrose and sorbitol (Table 2.6). Sucrose was found to be the most efficient carbon source and a combination of sucrose (30 g l^{-1}) and 2,4-D was far more effective for embryogenic callus induction than the other combinations. Although sorbitol (with 2,4-D) has been successful for other cultures (Ryschka *et al.*, 1991; Okamoto *et al.*, 1996), this sugar failed to stimulate callus formation in the explants of *C. dactylon* (Table 2.6). In some other studies an increase in sucrose concentration resulted in the suppression of non-embryogenic callus and the increased compactness of embryogenic callus (Elkonin *et al.*, 1995, Pedrosa and Vasil, 1996; Saidi *et al.*, 1997), the opposite was found to be true in this work.

In previous studies, plant regeneration of most grasses were achieved mainly by culturing somatic embryos on either a hormone-free culture medium (Chaudhury and Qu, 2000; Lauzer *et al.*, 2000) or on one supplemented with low levels of auxins and cytokinins (Wang *et al.*, 2003; Fei *et al.*, 2002; Bai and Qu, 2000; Saxena and Dhawan, 1999). However in this study, plant regeneration was achieved when partially-dried (3h) *C. dactylon* somatic embryos were cultured on hormone-free MS nutrient medium (Table 2.8). Since dehydrating mature zygotic embryos triggers germination and development into normal plants (Gray, 2000; von Arnold *et al.*, 2002), a similar approach was investigated for *C. dactylon* somatic embryos. A number of culture media components that decrease the water potential of the culture medium (e.g. high sucrose levels, ABA, PEG) were shown to promote somatic embryo maturation. However some of those maturation treatments have been shown to cause adverse effects on embryo quality, thereby impairing subsequent germination and *ex vitro* growth. Bozhkov and Arnold (1998) reported that although PEG stimulated somatic embryo maturation it also inhibited further development in the gymnosperm *Picea abies* by altering the organisation of the root meristem. In this study none of the *C. dactylon* somatic embryos that were exposed to either ABA or PEG germinated (Table 2.8). It is possible, therefore, that these agents inhibited the germination of the somatic embryos by disrupting further development, as suggested by Bozhkov and Arnold (1998).

In this work *C. dactylon* somatic embryos were dehydrated since dehydration followed by rehydration is known to trigger germination in zygotic embryos (Bewley and Black, 1985). Thus the physical drying treatment (3h) proved to be very efficient with a 100% of embryos germinating when cultured on a hormone-free MS nutrient medium (Table 2.8). Fehér *et al.* (2003) suggested that *in vitro* tissue culture conditions exposed explants to stresses (e.g. wounding, culture medium), thereby promoting de-differentiation and inducing somatic embryo formation. In this study the drying treatment probably acted as a trigger on the somatic embryo cells, inducing germination. Early studies found that physical drying treatments stimulated germination in *Dactylis glomerata* (Gray, 1987) and *Zea mays* (Compton *et al.*, 1992) and this approach was also used more recently for some gymnosperms (Capuana and Debergh, 1997; Norgaard, 1997). Physical drying treatments are not generally pursued for grass tissue culture since the standard approach (low levels of plant hormones or hormone-free) appears to stimulate germination in most studies.

2.4.2 Node culture

Although the organogenesis morphogenic pathway is not a common *in vitro* regenerative route for many grasses (Krishnaraj and Vasil, 1995), it was pursued for *C. dactylon* since it offers many advantages. These include the production of plants that are free of microorganisms (e.g. fungi, bacteria), a potential source of explant material for other *in vitro* cultures, true-to-type clones of the parent plant and the production of regenerants in a relatively short culture time. In this work lateral node buds were induced to break dormancy and produce shoots (Table 2.2). Further, a high level of cytokinin in the MS culture medium stimulated explants to produce multiple shoots. These shoots were rooted in a medium containing a low level of auxin. In this study plantlets were also produced using a single hormone-free medium (i.e. both shoots and roots developed in the same medium). These *in vitro*-produced plantlets were successfully acclimatised to the *ex vitro* conditions of the greenhouse.

2.4.3 Plant yield

In this work plants were propagated from nodal segments (20-30 mm long) via the organogenesis approach and both shoot and root induction was achieved in a single nutrient medium. Low plantlet yields are generally reported for the grasses when the organogenesis method of propagation is used (Table 2.9). The yield achieved for *C.*

dactylon in this study (1 plant/explant) is similar to those obtained for other grasses in earlier studies (Table 2.9). Although *C. dactylon* plant yields were low, this was achieved in a much shorter time period (10 days) than those earlier studies (4-8 weeks) (Table 2.9). In contrast to this and other earlier studies on the grasses, Flachsland *et al.* (1997) were able to increase the yield of *Setaria anceps* (7.6 shoots/node) by inducing shoot multiplication with a strong cytokinin. Similar attempts were made to increase *C. dactylon* plantlet yields in this study but a low percentage of the multiplied shoots formed roots (Table 2.2).

Table 2.9 Published yields of plantlet regeneration via nodal culture in the grasses

Species	Yield	Culture time	Reference
Cynodon dactylon	1 plant/explant	10 days	Present study
Setaria anceps	7.6 plants/node	60 days	Flachsland *et al.*, 1997
Panicum virgatum	1.2 shoots/node	8 weeks	Alexandrova *et al.*, 1996a
Bambusa glaucescens	0.51 plants/explant	6 weeks	Jullien and Tran Thanh Van, 1994
Poa pratensis	0.95 plants/explant	4 weeks	Pieper and Smith, 1988

All studies used a uninodal segment as the explant source.

As per reports in the literature, for *C. dactylon*, the somatic embryogenesis approach offers much higher yields (Table 2.10) than organogenesis (Table 2.9). However this regenerative route does require a much longer culture period (14 weeks) than the organogenic route (10 days). Further plant regeneration via somatic embryogenesis is obtained at a higher financial cost than organogenesis since in the former developmental route *C. dactylon* regeneration requires four stages and several culture media.

Table 2.10 Published yields of plantlet regeneration via somatic embryogenesis in the grasses

Species	Explant	Yield	Culture time	Reference
Cynodon dactylon	leaf base	198 germinating embryos/g fresh callus	14 weeks	Present study
	young inflorescence	80% of E callus formed plants	8-10 weeks	Chaudhury and Qu, 2000
Paspalum notatum	mature seeds	1640 plantlets/g fresh callus	11 weeks	Grando *et al.*, 2002
	leaf-stem sections	300 plantlets/g callus	?	Shatters *et al.*, 1994
Festuca arundinacea	mature seeds	22% of calli formed plants	12 weeks	Bai and Qu, 2000
Dendrocalamus strictus	mature seeds	90% of embryos germinated	11 weeks	Saxena and Dhawan, 1999
Eragrostis tef	immature embryo	92% of calli formed plants	12 weeks	Kebebew *et al.*, 1998
Panicum virgatum	mature caryopse	361 plantlets/E callus	90 days	Denchev and Conger, 1995
	leaf tissue	40-50 shoots/callus	60 days	
*Pennisetum americanum**	immature inflorescence	22 plants/E callus	?	Pius *et al.*, 1993
Agrostis palustris	mature seeds	40 plants/E callus	?	Zhong *et al.*, 1991
Pennisetum purpureum	young leaves	55-60 plants/g fresh E callus	30 weeks	Chandler and Vasil, 1984

E = embryogenic. **Pennisetum americanum* has been renamed *Pennisetum glaucaum* (Wang *et al.*, 2001).

Reports in the literature show that *in vitro*-produced plants are successfully acclimatised to *ex vitro* growth conditions and develop normally with no morphological differences when compared to seed grown plants (Kebebew *et al.*, 1998; Lauzer *et al.*, 2000; Aguado-Santacruz *et al.*, 2001; Grando *et al.*, 2002; Wang *et al.*, 2003). In fact the *in vitro* regenerants are some times found to have superior growth patterns than their seed derived counterpart, as shown by *Lolium perenne* regenerants which produced a significantly higher seed yield than the seed-grown plants (Stadelmann *et al.*, 1998). A similar finding was realised in this work with the *ex vitro* growth of the *C. dactylon* regenerants that were propagated *via* the organogenesis route (Table 2.3). In the first month these *in vitro*-produced plants were found to be much faster growing than their conventionally-produced counterpart and after 3 months there was no significant difference in the growth rate of these two plants and no visual difference. Similarly *C. dactylon* plants regenerated through somatic embryogenesis developed normally.

In conclusion, the *in vitro* regeneration of *C. dactylon* has been achieved through both the organogenesis and somatic embryogenesis developmental pathways, processes that are similar to other grasses. In the former morphogenic route plants were regenerated in 10 days from nodal segments that contained lateral buds. These explants were cultured in a single hormone-free MS nutrient medium that included 30 g l^{-1} sucrose and 10 g l^{-1} agar. The latter approach involved the induction of callus from leaf bases that were cultured on MS nutrient medium that contained 3 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose and 10 g l^{-1} agar. Callus was subcultured onto fresh nutrient media after 2 weeks and maintained on this medium for a further 4 weeks. Thereafter embryogenic calli were selected, dehydrated for 3 hours and transferred onto hormone-free MS nutrient medium containing 30 g l^{-1} sucrose and 10 g l^{-1} agar. Germinating somatic embryos were cultured onto fresh hormone-free nutrient media and acclimatised plants were transferred to *ex-vitro* conditions after 8 weeks. The plantlet yield from the established somatic embryogenesis protocol (198 germinating embryos/g fresh callus) was much higher than those obtained via organogenesis (1 plant/explant). Further the use of vegetative explant material (i.e. leaf base and nodal segment) ensures *in vitro* regeneration of *C. dactylon* throughout the year, unlike when reproductive explant material is used. To date *in vitro* regeneration has been reported for *C. dactylon* using the inflorescence (Ahn *et al.*, 1985) but not the leaf. The present study, therefore, makes an important contribution to the field of grass micropropagation since it offers the

potential use of a readily available vegetative explant source for the *in vitro* regeneration of this valuable turf and forage grass.

CHAPTER 3: DEVELOPMENT AND USE OF AN *IN VITRO* CALLUS CULTURE SYSTEM FOR SCREENING FOR Al^{3+} RESPONSE

3.1 Introduction

Root growth analysis in acidic culture media containing Al is an efficient and relatively simple method for identifying Al-resistant individuals (Taylor, 1988; Čiamporová, 2002; Samac and Tesfaye, 2003). Inhibition of root elongation results from a complex network of interactions between Al and components of the root cell. However, despite the advancement of many theories to explain the cause of Al toxicity in plants, the initial target sites and primary mechanisms of Al toxicity still remain unknown (Kochian, 1995; Matsumoto *et al.*, 2001). Many authors (Conner and Meredith, 1985ab; Parrot and Bouton, 1990; Yamamoto *et al.*, 1994; Espino *et al.*, 1998; Ikegawa *et al.*, 2000; Toan and Debergh, 2002) have investigated an alternative approach, the use of cell cultures, in order to improve the understanding of Al toxicity and resistance. In the present chapter a similar approach was investigated and the *in vitro* nutrient medium supporting callus growth was modified in order to provide an available supply of phytotoxic Al ion (Al^{3+}).

The most common approach to screening plants for resistance to Al is through root growth studies in one of three media containing Al; acid soils, sand and nutrient solutions. Table 3.1 presents details of studies using those media over the last decade. The majority of work has focused on the cereals. Acid soils have been used to screen several genotypes of, amongst other species, *Sorghum bicolor* (Baligar *et al.*, 1993); *Tripsacum dactyloides* (Foy, 1997); and *Triticum aestivum* (De Sousa, 1998). In those studies root length of plants in the limed and unlimed soils was evaluated and those plants showing the least inhibition in root growth in the unlimed soil were deemed as Al-R. Generally, the root length of the Al-S plants increased with the application of lime whereas root growth of the Al-R plants was similar in both soil treatments. Many researchers consider acid soils as a suitable medium for screening plants for Al-resistance since it represents most accurately the environment in which the selected Al-R individuals will be growing (Foy, 1997; De Sousa, 1998; Gallardo *et al.*, 1999). This approach is, however, faced with certain limitations. The Al and nutrient levels of the acid soils are difficult to measure and regulate and other soil components (e.g.

Table 3.1 Various published methods using root growth analysis to screen for Al-resistance

Type of culture	Species	Nutrient medium [μM]	pH	Al source	Time in culture	Speciation model	Reference
acid soil	*Sorghum bicolor*	dark red latosol, pH adjusted with $CaCO_3$	4.7-5.9	64% Al* saturation	28 d	-	Baligar *et al.*, 1993
acid soil	*Tripsacum dactyloides*	Tatum clay loam subsoil, fertilizer (N:P:K, 100:109:137 $\mu g\ g^{-1}$), pH adjusted with $CaCO_3$	4.4-5.7	Al 64-77%	13-17 w	-	Foy, 1997
acid soil (field)	*Triticum aestivum*	Haplorthox, ≈ 45% clay, P [24.2 ppm], K [131 ppm], Ca [1.07 cmol/l], Mg [0.38 cmol/l]	4.2-4.9	2.5-4.27 cmol/l	1 y	-	De Sousa, 1998
sand	*Picea abies*	NH_4 [300], NO_3 [300], Na [50.1], SO_4 [345.2], K [130], PO_4 [30], Mg [60], Ca [130], Mn [5], Fe [5], B [5], Mo [0.1], Zn [0.1], Cu [0.1], Cl [5]	3.9	$Al(NO_3)_3$ 800 μM	35 d	-	Jentschke *et al.*, 1991
nutrient solution	*Triticum aestivum*	Ca (400), Cl (425), K (650), NO_3 (730), Mg (250), NH_4 (80), roots stained for 40 min with 0.2% hematoxylin	4.2	$AlCl_3$ 50 μM	24 h	GEOCHEM**	Rincon and Gonzales, 1992
nutrient solution	*Triticum aestivum*	K (502), NO_3 (1250), Ca (500), NH_4 (250), Mg (125), SO_4 (125), PO_4 (2), Fe (2), Cl (10), B (10), Mn (2), Zn (4), Cu (2)	4.1	$AlK(SO_4)_2$ 100 μM	24 h	-	Delhaize *et al.*, 1993a
nutrient solution	*Tritcale spp*	Ca [4000], NO_3 [8000], K [4500], Mg [2000], SO_4 [2437], PO_4 [500], NH_4 [435], Zn [0.8], Na [30], Cl [30], Mo [0.1], B [10], Fe [10], Mn [2], Cu [0.3]	4	$Al_2(SO_4)$ 5-20 μM	2 d	-	Antunes *et al.*, 1996

Table 3.1 contd.

Type of culture	Species	Nutrient medium [μM]	pH	Al source	Time in culture	Speciation model	Reference
nutrient solution	*Glycine max*	Ca (800) SO_4 (800)	4.3	$AlCl_3$ 1.2-19 μM	3 d	GEOCHEM	Bianchi-Hall *et al.*, 1998
nutrient solution	*Zea mays*	Ca (200), Mg (100), K (400), NH_4 (300), NO_3 (700), SO_4 (315), Zn (0.38), Mn (15), Fe (10), EDTA (10), Na (5), PO_4 (5), Cu (0.16), Mo (0.06)	4.4	$AlCl_3$ 30 μM	24 h	GEOCHEM	Pintro *et al.*, 1998
nutrient solution	*Glycine max*	Ca (200), Cl (200), roots stained with 10 μM lumogallion for 1 h	4.4	$AlCl_3$ 50 μM	1 h	-	Kataoka and Nakanishi, 2001
nutrient solution	*Oryza sativa*	Ca [100], Cl [100]	4.01	$AlCl_3$ 640	48 h	-	Vasconcelos *et al.*, 2002
		Ca [175], Mg [667], K [430], N-NH_4^+ [264], N-NO_3^- [2130], S [108], Cl [107], P [6.5], Fe [11.6], B [4.2], Mn [1.5], Zn [0.38], Mo [0.16], Cu [0.15]	4.01	80-320 μM	9 d	-	
sand	*Glycine max*	Ca (4000), SO_4 (4270), K (252000), PO_4 (250000), NO_3 (2000), Fe (18), Cl (18.9), B (9.3), Mn (0.9), Zn (0.9), Cu (0.18), Mo (0.18), Mg (250)	4.3	$AlCl_3$ 800 μM	11 d	GEOCHEM	Villagarcia *et al.*, 2001
nutrient solution	*Glycine max*	Ca [800], SO_4 [800]	4.3	Al^{3+} {2.5 μM }	3 d	GEOCHEM	Villagarcia *et al.*, 2001

Table 3.1 contd.

Type of culture	Species	Nutrient medium [μM]	pH	Al source	Time in culture	Speciation model	Reference
nutrient solution	*Hordeum vulgare*	$N\text{-}NO_3^-$ [3710], $N\text{-}NH_4^+$ [310], Ca^{2+} [1270], K^+ [750], $S\text{-}SO_4^{2-}$ [120], $P\text{-}HPO_4^{2-}$ [100], Fe [17.9], B [6.6], Mn [2.4], Zn [0.6], Cu [0.2], Mo [0.1]	4.8-6	$AlK(SO_4)_2$ 50-200 μM	15 d	-	Gallardo *et al.*, 1999
acid soil		Gorbea soil, typical Andisol with highly exchangeable Al, limed with $CaCO_3$	4.95-5.6	Al 3.1-21.6%	6 d	-	Gallardo *et al.*, 1999
acid soil (field)		non-acidic Cajon soil (pH 5.8), acidic Gorbea soil (pH 4.9), P [63], N [60], K [42], Mg [6] kg ha^{-1} applied to both sites	4.9-5.8	Al 1.2-21.6%	?	-	Gallardo *et al.*, 1999

*Aluminium (Al) source in soil not specified and Al saturation values 64% of CEC. GEOCHEM** Al speciation model (Parker *et al.*, 1995).

$\{Al^{3+}\}$ = Al activity predicted by model. y = year, w = weeks, d = days, h = hours.

organic matter) could bind and reduce Al availability. Screening plants in nutrient solution culture or sand supplemented with nutrients can potentially overcome those limitations (Jentschke *et al.*, 1991; Rincon and Gonzales, 1992; Gallardo *et al.*, 1999; Villagarcia *et al.*, 2001). In sand culture the sand is acid-washed in order to remove clay-bound particles and organic matter, and known amounts of nutrients and Al are added. This method has been used to select Al-R *Picea abies* (Jentschke *et al.*, 1991) and *Glycine max* (Villagarcia *et al.*, 2001).

The disadvantage of a solid medium such as soil and sand is the problems associated with root damage when the roots are harvested and the difficulty of making repeated root measurements. Thus, root growth in nutrient solutions containing Al is usually the preferred choice of many researchers (Rincon and Gonzales, 1992; Delhaize *et al.*, 1993a; Antunes *et al.*, 1996; Bianchi-Hall *et al.*, 1998; Pintro *et al.*, 1998; Vasconcelos *et al.*, 2002). Seedling roots are exposed to nutrient solutions at a low pH (< 4.5) that included or did not include Al. The length of the Al-treated and control roots are compared and those plants showing the lowest inhibition in root elongation are selected as Al-R. This approach allows for the measurement of root growth easily and nutrient and Al levels, and pH can be regulated. Further, the response time for an Al-induced effect on root growth in nutrient solutions was much shorter than for acid soils. For example, the time required to screen Al-R plants on acid soils ranged from 28 d (Baligar *et al.*, 1993) to 1 y (De Sousa, 1998) whereas the same can be achieved in nutrient solution after 1 h (Kataoka and Nakanishi, 2001). Nutrient solutions have been used to identify Al-R genotypes of, *inter alia*, *Triticum aestivum* (Delhaize *et al.*, 1993a), *Triticale* spp (Antunes *et al.*, 1996), *Glycine max* (Bianchi-Hall *et al.*, 1998), *Zea mays* (Pintro *et al.*, 1998) and *Oryza sativa* (Vasconcelos *et al.*, 2002). This method offers a simple and rapid means of screening many genotypes for Al-resistance.

Some researchers have evaluated, comparatively, the observed Al-resistance ranking obtained for plants screened in various ways in nutrient solutions, acid soils in pots, acid soils in the field and sand (Liu *et al.*, 1995; Gallardo *et al.*, 1999; Toda *et al.*, 1999; Villagarcia *et al.*, 2001). Gallardo *et al.* (1999) reported that in barley the Al-resistance

ranking estimated for each growth medium tested was the same. Those authors suggested therefore that laboratory-based short-term screening methods provided a useful tool for the selection of Al-R plants for use in the field. In contrast Villagarcia *et al.* (2001) reported that hydroponics-based soybean seedling screening resulted in an Al tolerance ranking that differed from those obtained through root growth in sand. They suggested that although screening seedlings in nutrient solution is a simple and rapid means of selecting Al-T individuals, their tolerance ranking should be validated with older plants and in solid media.

Monitoring seedling root growth in a nutrient solution containing the essential macro- and micronutrients together with Al is the usual approach for screening for Al-resistance. In order to maximise Al availability in this culture medium some researchers have modified the composition of the plant growth nutrient solution. A chemical speciation program (GEOCHEM, Parker *et al.*, 1995) has been used to model the interactions of Al with elements in the plant growth nutrient solution and those elements predicted to chelate or precipitate Al have been reduced or removed (Rincon and Gonzales, 1992; Bianchi-Hall *et al.*, 1998; Pintro *et al.*, 1998; Villagarcia *et al.*, 2001). For example EDTA is known to chelate Al, and PO_4^{2-} combines with Al to form a precipitate, both agents reducing Al availability in solution (Andersson, 1988). Further, the cations Ca^{2+}, Mg^{2+}, K^+ and Na^+ have been reported to ameliorate Al toxicity (Kinraide and Parker, 1987b; Silva *et al.*, 2001; Kinraide, 2003). Some researchers used a simple salt medium ($CaSO_4$ or $CaCl_2$) in order to maximise Al availability and avoid the ameliorative effects of other elements (Bianchi-Hall *et al.*, 1998; Villagarcia *et al.*, 2001; Kataoka and Nakanishi, 2001; Vasconcelos *et al.*, 2002).

In addition to root growth, other means of identifying Al-R individuals include the use of Al stains (hematoxylin, morin and lumogallion). This method is based on the theory that Al-R plants are able to exclude Al from their roots, whereas the Al-S plants have inefficient Al exclusion mechanisms and take up more Al into their root cells than the Al-R plants (Kochian, 1995; Rengel, 1996; Matsumoto *et al.*, 2001; Barceló and Poschenrieder, 2002). Each of the Al stains forms a complex with Al such that roots with a high Al content (Al-S)

stain more intensely than roots with a low Al content (Al-R). Hematoxylin staining of roots was initially used as a rapid and simple method for the visual detection of Al tolerance in several wheat varieties (Polle *et al.*, 1978). Later studies used the hematoxylin stain, in addition to root growth studies in nutrient solutions, to confirm Al-R rankings in wheat (Rincon and Gonzales, 1992), perennial ryegrass (Bennet, 1995), sorghum (Yoshida and Yoshida, 2000) and barley (Echart *et al.*, 2002). In recent studies the fluorescent stains lumogallion (Kataoka and Nakanishi, 2001) and morin (Brauer, 2001) have been used to investigate Al distribution within the root cells of Al-S and Al-R genotypes of wheat and soybean, respectively.

Table 3.2 provides a summary of published studies in which *in vitro* techniques have been used to screen for Al-resistance. Early work in this field includes the screening of cell cultures of *Lycopersicon esculentum* (Meredith, 1978), *Daucus carota* (Ojima and Ohira, 1983) and *Sorghum bicolor* (Smith *et al.*, 1983) for Al resistance. In those initial studies the *in vitro* nutrient media (CS5, R2 and MS, respectively) were not modified to maximise free Al^{3+} ion activity, Al was supplied as Al-EDTA and the pH of the medium was not decreased to a pH < 6 (Meredith, 1978; Smith *et al.*, 1983). Conner and Meredith (1985a,b) developed a culture medium for *Nicotiana plumbaginifolia* that minimised Al precipitation and increased Al toxicity. The components of the nutrient medium that chelated the free Al^{3+} ions were modified, e.g. decrease in pH from 6 to 4, PO_4^{2-} reduced to 10 μM, Ca^{2+} reduced to 100 μM and EDTA was removed. Since 1985 similar modifications have been adopted by other researchers for MS nutrient media in investigations that screen for Al resistance (Campbell *et al.* 1989; Yamamoto *et al.*, 1994; Van Sint Jan *et al.*, 1997; Espino *et al.*, 1998; Martinez-Estevez *et al.*, 2001).

In some recent studies, a simple salt solution (i.e. $CaCl_2$) containing Al has been used as the Al-screening medium (Devi *et al.*, 2001; Zhu *et al.*, 2003). As in the screening of whole plants, the use of a simplified salt medium eliminates the interaction of Al with the various components of the complete nutrient medium, maximises free Al^{3+} ion activity and reduces culture time (Devi *et al.*, 2001; Zhu *et al.*, 2003). Further, it appears that regardless of type of species, screening in liquid nutrient media (i.e. cell suspensions) requires shorter culture

Table 3.2 *In vitro* approaches to screening for Al-resistance

Type of culture	Species	Screening medium [μM]	pH	Time in culture	Al source	[Al] [μM]	Reference
cell suspension	*Daucus carota*	R2 medium	4.5-4.7	20 w	$AlCl_3$ AlEDTA	4000 10000	Ojima and Ohira, 1983
	Nicotiana plumbaginifolia	MS modified: $CaCl_2$ [100], KH_2PO_4 [10], no EDTA	4	10 d	$Al_2(SO_4)_3$	200-400	Conner and Meredith, 1985a
	Nicotiana tabacum	MS modified: no PO_4 and EDTA	4	21 h	$AlCl_3$	100	Yamamoto *et al.*, 1994
	Nicotiana tabacum	$CaCl_2$ [3000]	4.5	24 h	$AlCl_3$	25-100	Devi *et al.*, 2001
	Coffea arabica	MS (half ionic strength)	4.3	24 d	$AlCl_3$	25-1000	Martinez-Estevez *et al.*, 2001
	Picea rubens	Litvay medium (half ionic strength)	4.2	24-48 h	$AlCl_3$	200-1000	Minocha *et al.*, 2001
	Hordeum vulgare	$CaCl_2$ [200]	4.5	24 h	$AlCl_3$	30	Zhu *et al.*, 2003
callus	*Lycopersicon esculentum*	CS5 medium	5.9-6	28 d	AlEDTA	10000	Meredith, 1978
	Sorghum bicolor	MS	?	2-3 m	$Al_2(SO_4)_3$	100-400	Smith *et al.*, 1983

Table 3.2 contd.

Type of culture	Species	Screening medium [μM]	pH	Time in culture	Al source	[Al] [μM]	Reference
	Glycine max	MS modified: NO_3 [15000], NH_4 [15000], Ca [1500], K [13500], Zn [85000], no EDTA	4.6	?	$Al_2(SO_4)_3$	2500	Campbell *et al.*, 1989
	Medicago sativa	Blaydes modified: PO_4 [10], Ca [100], no EDTA	4	8 w	$AlCl_3$	400	Parrot and Bouton, 1990
	Oryza sativa	MS modified: NH_4NO_3 [30000], $MgSO_4$ [1500], $CaCl_2$ [100], KH_2PO_4 [100], $FeSO_4$ [100], no EDTA	3.85	20 w	$Al_2(SO_4)_3$	2000	Van Sint Jan *et al.*, 1997
	Phaseolus vulgaris	MS salts and B5 vitamins modified: PO_4 [10], Ca [100], no EDTA	4	4 w	$AlCl_3$	2071	Espino *et al.*, 1998
	Citrus sinensis *C. aurantium*	MS modified: NH_4NO_3 [30000], $MgSO_4$ [1500], $CaCl_2$ [100], KH_2PO_4 [100], $FeSO_4$ [100], no EDTA	3.85	4 w	$Al_2(SO_4)_3$	400-2000	Toan and Debergh, 2002
anther	*Triticum aestivum*	MN_6 medium	4.2	30 d	$AlCl_3$	400	Karsai *et al.*, 1994

R2 (Ojima and Ohira, 1980), MS (Murashige and Skoog, 1962), Blaydes medium (Saunders and Bingham, 1975), B5 (Gamborg *et al.*, 1968), MN_6 (Chu *et al.*, 1990), Litvay medium (Litvay *et al.*, 1981), CS5 medium (Meredith, 1978). d = days, h = hours, m = months, w = weeks.

times for the selection of Al-R individuals than when solid gelatinous nutrient media (i.e. callus cultures) are used. Screening times reflected in Table 3.2 for various studies show that when cell suspensions are used the time ranges from 21 h to 10 d, with the exception of *Daucus carota* (Ojima and Ohira, 1983), whereas the screening times for callus cultures ranges from 4 w to 3 m.

In addition to screening for pre-existing resistance to Al, cell culture systems have also been used to induce resistance to Al through somaclonal variation. In an early study Ojima and Ohira (1983) induced Al-resistance in *Daucus carota* cell lines from an Al-S parental line by exposing the carrot cells to a high $AlCl_3$ concentration (4000 μM) for long culture intervals (8-20 w). A similar approach was adopted by Van Sint Jan *et al.* (1997) for the induction of Al-resistant *Oryza sativa* cultivars. In that study the Al-stress (1000 μM $Al_2(SO_4)_3$ for 20 w) was applied to callus and regenerated plants. However, among the three Al-R rice cultivars selected, one was isolated from the Al-free medium. Those authors suggested that the intensity of the Al-stress is not critical for isolating resistant individuals. It is not surprising that Al-R genotypes were induced on an Al-free medium since in the absence of the Al stress, other factors such as time in culture, nutrient and water stress can also influence changes at the genetic level. In another study, Al tolerance was induced in an Al-S barley cell line through the use of mutagens (e.g. ethyl methane sulfonate) (Zhu *et al.*, 2003). Those authors selected four Al-T barley cell lines in a simplified salt solution containing 30 μM $AlCl_3$.

The aim of this study was to assess the feasibility of using meristematic callus cells to investigate Al^{3+} toxicity and resistance. The objective of the present chapter was to develop the *in vitro* nutrient medium that contained the essential nutrients required for callus growth as well as an available Al^{3+} supply. The nutrient medium was established in the somatic embryogenesis protocol (chapter 2) and modified in order to provide a known supply of free Al^{3+} ions. Relative Al^{3+} toxicity was measured using growth and therefore any reductions in nutrient levels of the *in vitro* medium had to be minimised to allow for active callus growth.

3.2 Materials and Methods

3.2.1 Plant material

Three *C. dactylon* genotypes were used in this study. These genotypes were selected from seed (section 2.2.1), acidic mine tailings and a commercial turfgrass nursery (Top Crop Nursery, Cramond, South Africa). Referred to as Genotype 1, 2 and 3, respectively. A single parent plant of each genotype was selected and mass propagated in the greenhouse through macrocuttings, each comprising a node with axillary buds and root primordia. Plant growth conditions have been mentioned in chapter 2 (section 2.2.1).

3.2.2 Callus induction

Callus was initiated from leaf bases that were cultured on MS nutrient medium containing 30 g l^{-1} sucrose, 10 g l^{-1} agar and 3 mg l^{-1} 2,4-D (pH 5.6-5.8), established as part of the somatic embryogenesis protocol (see section 2.2.3). These cultures were maintained in the dark in the growth room (25 °C day/18 °C night) and subcultured onto fresh nutrient medium after 2 weeks. Four week-old callus was transferred onto Al screening media.

3.2.3 Statistical analyses

The statistical test applied for data collated from each experiment is specified in the respective results figures and tables. Further, in all statistical tests significance was assessed using $p < 0.05$.

In experiments that examined the effect of a single factor, a One-Way Analysis of Variance (ANOVA) was run (Zar, 1984). The Statgraphics Plus Statistical Graphics System Version 7.0, computer software produced by Manugistics Inc. and Statistical Graphic Corporation, was used. The data (callus growth rate) were initially tested for normality using the Kolmogorov-Smirnoff test ($p > 0.05$). If significant differences were concluded among the variables tested then the Scheffé's multiple comparison test was applied.

A multifactor ANOVA was used to analyse callus growth rate data when the simultaneous influence of more than one factor was being assessed (Zar, 1984). This test was run using SPSS Advanced Statistics (Release 9.01) computer software produced by SPSS Inc.

(Norušis, 1994). The callus growth rate data were transformed (square root) in order to achieve a normal distribution and the One-sample Kolmogorov-Smirnov normality test ($p > 0.05$) was applied to the residuals of the transformed data. The multifactor ANOVA examined effects of the three *C. dactylon* genotypes, five Al^{3+} concentrations and four culture times on callus growth rate. If significant differences were concluded among levels of a single factor then the Scheffé's multiple comparison test was applied.

To examine differences in the incidence of embryogenic versus non-embryogenic callus, a Chi-Squared test was used (Zar, 1984). This test is usually applied on nominal scale data when the influence of a single factor is being examined. The relationship among the five Al^{3+} concentrations at each culture interval was evaluated by assessing the counts obtained from scoring the callus as either embryogenic or non-embryogenic.

A Two-way ANOVA (SPSS) was run on transformed callus growth rate data, examining the interaction between genotype and Al^{3+} treatment, using only the 2 week data. In the literature most researchers selected the lowest Al^{3+} concentration and the shortest culture time for screening genotypes for resistance to Al. Two weeks was, therefore, selected and a Scheffé's multiple comparison test was used to determine the lowest Al^{3+} concentration at which callus growth rate is significantly different from the control in all three genotypes. Further tests on 4 week data were not performed as the 2 week data yielded significant relationships.

3.2.4 Development of Al^{3+} screening medium

MINTEQA2

A geochemical speciation model (MINTEQA2), developed by the United States Environmental Protection Agency, was used in this study (Allison *et al.*, 1991). The interactive part of the program, PRODEFA2, is used to create input files. This modeling system has an extensive database of thermodynamic data pertaining to reactions leading to chemical complex formation, mineral dissolution/precipitation, gas absorption and redox reactions. The second part of the program, Xminteqx, uses the database to create the output files that were accessed with MS Word (Version 6.0). The input data parameters of the

model were: temperature (set at 25°C); molar concentration of ionic species; precipitation (over-saturated components were allowed to precipitate when observed in experiments regarding Al^{3+} interactions in the MS and modified nutrient media but was not set for $AlCl_3$ and $Al_2(SO_4)_3$ hydrolysis reactions); the maximum number of iterations was set at 200; and the pH was set accordingly.

Aluminium fractionation

As mentioned earlier, the Al^{3+} ion has been identified as the phytotoxic Al species responsible for inhibited root elongation (section 1.4). The interactions of this ion were first modelled in water at different pH values. The chemistry of two aluminium salts [$AlCl_3$ (0.1 M) and $Al_2(SO_4)_3$ (0.05 M)] in solution was modeled using MINTEQA2 (Figure 3.1A, B). Several aluminium hydrolysis species were formed with increasing pH and the fractionation species predicted by MINTEQA2 were similar to those proposed in other earlier work, presented in chapter one (Smith, 1971; Driscoll and Schecher, 1990). The Al^{3+}, $Al(OH)_3$, $AlOH^{+2}$, $Al(OH)^{2+}$, $Al(OH)_4^-$ species are common to both aluminium salts, while $AlSO_4^+$ and $Al(SO_4)_2^-$ only result from the use of $Al_2(SO_4)_3$. At pH values less than and including 5, the Al^{3+} ion predominates with $AlCl_3$ (Figure 3.1A). Further, as the solution becomes less acidic (i.e. pH > 5) the Al^{3+} ion is deprotonated to yield $Al(OH)^{2+}$ and $Al(OH)_2^+$. At a neutral pH most of the aluminium supplied is available as $Al(OH)_3$ and ($Al(OH)_4^-$). In contrast, at low ($\leq$ 5) pH values $Al_2(SO_4)_3$ is fractionated into Al^{3+}, $AlSO_4^+$ and $Al(SO_4)_2^-$ (Figure 3.1B). Similar to $AlCl_3$, approximately 60% of the aluminium supplied forms an aluminate ion ($Al(OH)_4^-$) at pH 7. MINTEQA2 predictions therefore show that for the same aluminium concentration (i.e. 0.1 M Al), $AlCl_3$ provides a greater source of Al^{3+} ions than $Al_2(SO_4)_3$ (Figure 3.2). The reason for this is that at low pH $Al_2(SO_4)_3$ yields three Al species ($AlSO_4^+$, $Al(SO_4)_2^-$ and Al^{3+}), unlike $AlCl_3$.

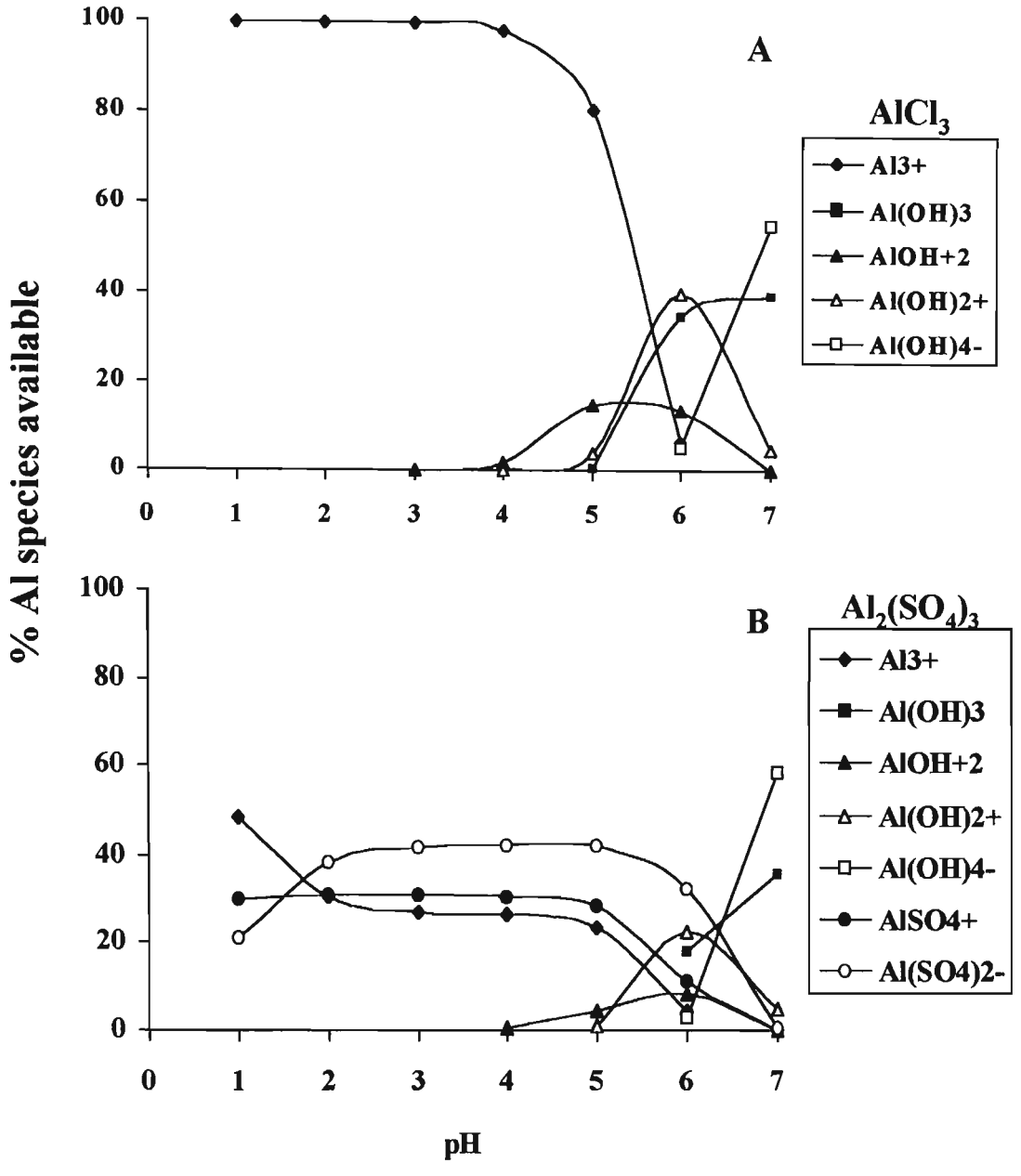

Figure 3.1 Distribution of soluble, mononuclear aluminium ion species in water as a function of pH. The aluminium fractionation models for $AlCl_3$ (A) and $Al_2(SO_4)_3$ (B) were predicted using MINTEQA2. Total Al supplied (0.1 M). The model was set such that over-saturated components did not form a precipitate.

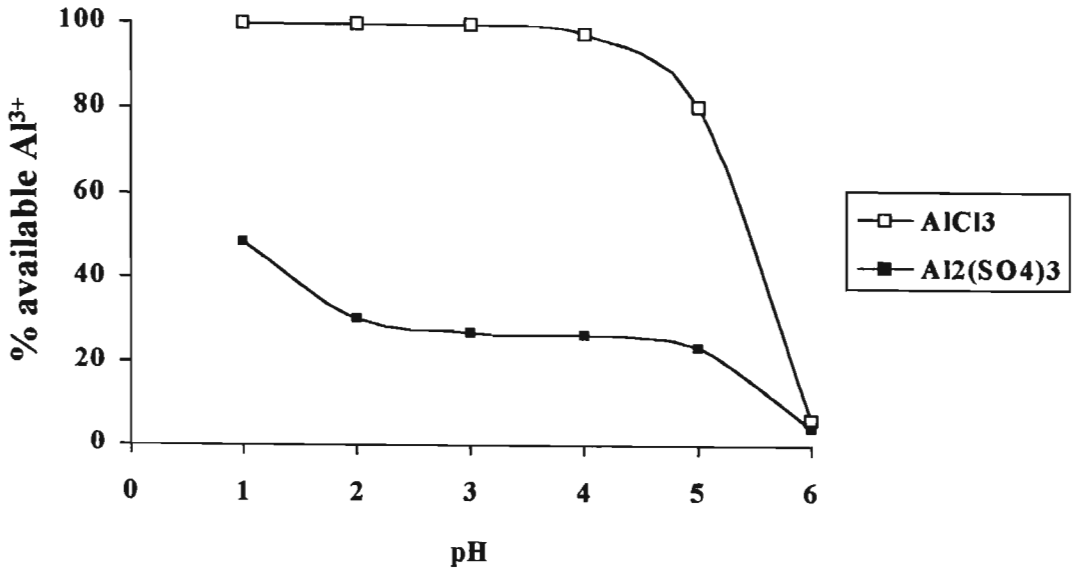

Figure 3.2 A comparison of Al^{3+} ion availability between two aluminium salts. $AlCl_3$ and $Al_2(SO_4)_3$ were supplied at 0.1 M (Al) and Al^{3+} availability was calculated using MINTEQA2 predictions at various pH levels. The model was set such that over-saturated components did not form a precipitate.

Al^{3+} assay: An assessment of the MINTEQA2 speciation model

The catechol violet colorimetric method (Wilson, 1984) was used to measure the Al^{3+} ions in solutions of $AlCl_3$ and $Al_2(SO_4)_3$. Glassware washed with 1 M HCl was used throughout. A range (0.03-1 M) of $AlCl_3$ and $Al_2(SO_4)_3$ standards were prepared in HCl (0.1 M, pH 0.85-0.92) and a standard curve was calculated for each aluminium salt. Solutions (in water) of $AlCl_3$ (0.5-16 mM) and $Al_2(SO_4)_3$ (0.25-8 mM) were set at pH 3.5. The sample or standard solution (0.25 ml) was added to a borosilicate glass tube containing 10 ml HCl (0.1 M). The solution was mixed well with a vortex-type mixer following the addition of the following reagents: 0.25 ml iron-reducing complexing solution ($NH_2OH.HCl$ (1.4 M) and $C_{12}H_8N_2.H_2O$ (5 mM)); 0.5 ml catechol violet solution (0.4 g l^{-1}); and 2.5 ml buffer solution (NH_4OH (0.26 M)) and $C_6H_{12}N_4$ (2.5 M)). The final solution (pH 6.18-6.22) was incubated for 30 min at room temperature and the absorbance was measured in a Beckman DU 500 spectrophotometer at 585 nm, using glass cuvettes (10 cm light path). The

measured Al concentrations compared favourably with the Al^{3+} concentrations predicted by MINTEQA2 (Table 3.3). It seems, therefore, that the MINTEQA2 model provides an accurate representation of aluminium speciation, and in particular the availability of the free Al^{3+} ions. Also, since $AlCl_3$ provides a greater source of free Al^{3+} ions than $Al_2(SO_4)_3$, this aluminium salt was used in all subsequent experimental investigations in this study.

Table 3.3 Predicted Al^{3+} concentrations (MINTEQA2) and assayed Al^{3+} ions (catechol violet method) in two aluminium salt solutions, $AlCl_3$ and $Al_2(SO_4)_3$, set at pH 3.5

Selected	$AlCl_3$		$Al_2(SO_4)_3$	
[Al]	Predicted Al^{3+}	Assayed Al^{3+}	Predicted Al^{3+}	Assayed Al^{3+}
mM	mM	mM	mM	mM
0.5	0.49	0.54 ± 0.015	0.37	0.29 ± 0.0002
1	0.97	1.01 ± 0.16	0.66	0.51 ± 0.003
2	1.95	1.95 ± 0.01	1.17	1.01 ± 0.002
4	3.92	3.97 ± 0.02	2.07	2.05 ± 0.002
8	7.9	7.68 ± 0.01	3.69	4.05 ± 0.003
16	15.85	16.6 ± 0.03	6.76	7.35 ± 0.01

Mean assay value ± SE, n = 6.

Modeling Al^{3+} availability in published in vitro *culture media*

Seven of the published *in vitro* culture media mentioned in Table 3.2 of the introduction (section 3.1) have been evaluated with regard to Al^{3+} activity using MINTEQA2. The chemical interactions of aluminium were modeled with the components of the nutrient media and the resulting Al^{3+} activity was predicted with increasing aluminium supply (Figure 3.3).

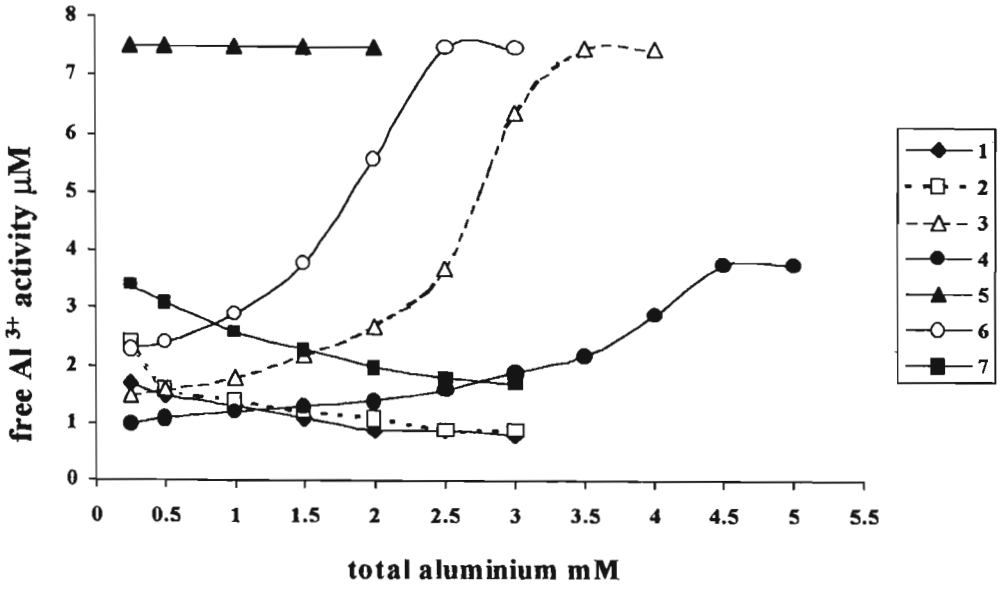

Figure 3.3 MINTEQA2 simulation of published media used to screen *in vitro* cultures for aluminium tolerance. All media at pH 4, except medium 7 (pH 3.85). Media 1–7 are: **1**. Conner and Meredith (1985a): MS modified (0.1 mM $CaCl_2$, 0.01 mM KH_2PO_4, 0 mM EDTA), $Al_2(SO_4)_3$; **2**. Campbell *et al.* (1989): MS modified (8.7 mM NH_4NO_3, 13.5 mM KNO_3, 0.085 mM $ZnSO_4$), $Al_2(SO_4)_3$; **3**. Karsai *et al.* (1994): wheat anther culture medium, $AlCl_3$; **4**. Koyama *et al.* (1995): B5 modified (40 mM KNO_3, 0 mM EDTA, 0.1 mM NaH_2PO_4), $AlCl_3$; **5**. Dall'Agnol *et al.* (1996): Blaydes medium modified (0 mM K_2HPO_4, 0 mM EDTA) $AlCl_3$; **6**. Yamamoto *et al.* (1996): MS modified (0 mM KH_2PO_4, 0 mM EDTA), $AlCl_3$; **7**. Van Sint Jan *et al.* (1997): MS modified (0.1 mM KH_2PO_4, 30 mM NH_4NO_3, 1.5 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1 mM $FeSO_4$), $Al_2(SO_4)_3$.

The molar concentrations of the ionic species of the various components of each of those formulations and a range of aluminium (either as $AlCl_3$ or $Al_2(SO_4)_3$) were entered into PRODEFA2. The program was set to run at pH 4, except for medium 7 (pH 3.85), and over-saturated components were allowed to precipitate. Simulations of the MINTEQA2 model showed that, using the tested media, the maximum Al^{3+} activity reached is 7.5 µM, corresponding to an aluminium concentration of 2.5 mM for medium 6 (Yamamoto *et al.*, 1996) and 3.5 mM for medium 3 (Karsai *et al.*, 1994). With media 3 and 6, as Al^{3+} activity increases, increasing amounts of alunite ($KAl_3OH(SO_4)_2$) are produced (0.05 - 0.9 mM for 0.25 - 3.5 mM $AlCl_3$), explaining the discrepancy between the concentration of aluminium in the medium and Al^{3+} activity. At concentrations higher than 2.5 mM for medium 6 and

3.5 mM for medium 3 (when the Al^{3+} activity plateau is reached) another precipitate, diaspore (α-$Al_2O_3.H_2O$), is formed. The concentration of this compound is 0.3 mM for medium 6 and 0.4 mM for medium 3, when first detected, and increases with $AlCl_3$ supply. The activity of Al^{3+} remains constant as soon as diaspore is formed. With the formulation of Dall'Agnol *et al.* (1996) (medium 5), maximum activity (7.5 µM) and diaspore formation occur at 0.25 mM $AlCl_3$ as these authors specifically altered the medium, by decreasing the amount of sulphate (0.3 mM), to use a low aluminium concentration (0.4 mM). The highest possible Al^{3+} activity with medium 4 (Koyama *et al.* 1995) is 3.8 µM, reached at 4.5 mM $AlCl_3$ (Figure 3.3). This is due to this medium containing higher levels of SO_4^{2-} (3.4 mM) and K^+ (40 mM) than media 3 (2.1 mM SO_4^{2-} and 15.5 mM K^+) and 6 (1.7 mM SO_4^{2-} and 18.8 mM K^+). Hence, more alunite is formed in medium 4 (0.2 mM - 1.2 mM, for 0.25 mM - 3.5 mM $AlCl_3$) than in the others, removing the Al^{3+} from solution.

With the other media (1, 2, and 7), increases in aluminium concentration do not result in concomitant increases in aluminium activity (Figure 3.3). Those media were used by Conner and Meredith (1985a) (medium 1), Campbell *et al.* (1989) (medium 2) and Van Sint Jan *et al.* (1997) (medium 7) who supplied aluminium as $Al_2(SO_4)_3$. Consequently, in those formulations, increased aluminium and SO_4^{2-} supply are coupled resulting in increasing alunite formation [0.1 - 1.9 mM, for 0.25 mM - 3 mM $Al_2(SO_4)_3$] rather than Al^{3+} activity. No diaspore is formed under those conditions.

Modification of the callus culture medium

In the present study the Murashige and Skoog (1962) nutrient formulation is referred to as the standard MS culture medium. Conner and Meredith (1985a) established that several modifications to the standard MS nutrient medium are required to permit the availability of Al^{3+} in solution and to maximise aluminium toxicity in plant cell cultures. Those include: a reduction in pH from 5.6 to 4; the use of unchelated iron; and reduced phosphate and calcium concentrations. Those modifications were applied to medium MM3, which was compared to the standard MS nutrient media at pH 5.6 (MM1) and pH 4 (MM2), using MINTEQA2 (Figure 3.4). Aluminium was supplied as $AlCl_3$ (0.25-3 mM).

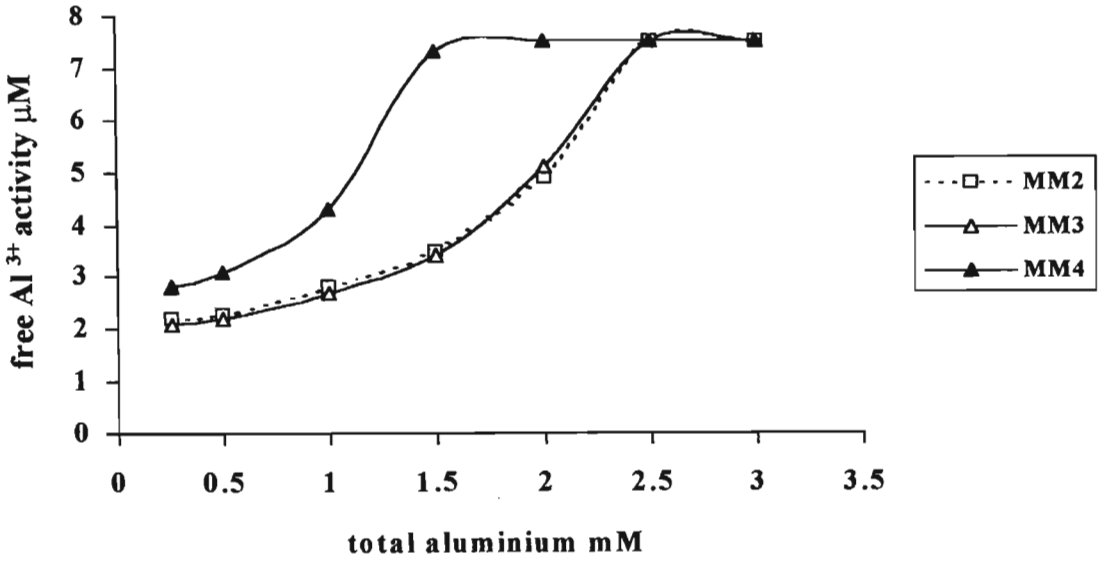

Figure 3.4 MINTEQA2 simulation of media (with $AlCl_3$) formulated for the screening of aluminium resistant genotypes of *C. dactylon*. All media at pH 4. MM2: MS standard; MM3: MS modified (0.1 mM $CaCl_2$, 0.01 mM KH_2PO_4, no EDTA); MM4: MS modified (1 mM $MgSO_4$, no EDTA). Note: in MM1 Al^{3+} activities were below 0.12 nM.

The simulations confirmed that Al^{3+} activity is very low (0.12 nM) on standard MS medium at pH 5.6 (MM1; Al^{3+} activities too low to be represented in Figure 3.4). The reason for this is that Al^{3+} availability is reduced at a pH greater than 5 and the Al precipitate diaspore is formed at the lowest $AlCl_3$ (0.25 mM) concentration tested. At pH 4 (medium MM2), Al^{3+} activity increased with increasing aluminium concentration, from 2 µM at 0.25 mM $AlCl_3$ to 7.5 µM at 2.5 mM $AlCl_3$. The relationship between Al^{3+} activity and increasing Al supply is similar in media MM2 and MM3. Medium MM3, formulated according to the recommendations of Conner and Meredith (1985a), contains less PO_4^{2-} (0.01 mM) than the standard MS medium (MM2) (1.2 mM PO_4^{2-}). The PO_4^{2-} ion interacts with several ions (H^+, Mg^{2+}, Ca^{2+}, Na^+, K^+ and Fe^{2+}) that are present in the standard MS medium, thus reducing its activity. The MINTEQA2 simulations showed that in the MS medium only 0.015 pM and 0.016 nM PO_4^{2-} are available at pH 4 and pH 5.6, respectively. At these very low PO_4^{2-} concentrations and pH 4, chelation between aluminium and

phosphate was not detected. Further MINTEQA2 simulations also showed no interaction between Al^{3+} and Ca^{2+}, even though a high concentration of Ca^{2+} (2.9 mM) is present in the standard MS medium. Since it has been established in this work that *C. dactylon* callus induction medium requires a high level of phosphate and calcium (chapter 2), it was decided to model a new modified MS medium (MM4) where these ions were not lowered.

In medium MM4 the levels of SO_4^{2-} (1 mM) and EDTA (0 mM) were altered since both these ions interacted with Al^{3+}. The level of SO_4^{2-} was reduced because in the standard MS medium SO_4^{2-} (1.7 mM) at pH 4 interacts with Al^{3+} in the culture medium producing 0.2 - 0.7 mM alunite (0.25 - 3 mM $AlCl_3$). Therefore, SO_4^{2-} was reduced to 1 mM in medium MM4 in an attempt to increase Al^{3+} activity through reducing the production of alunite. This resulted in increased Al^{3+} activity and decreased alunite production (0.08 – 0.5 mM) at all tested $AlCl_3$ concentrations up to 2 mM, when diaspore was formed (Figure 3.4). These were the highest Al^{3+} activities obtained for that range of $AlCl_3$ concentrations for all tested published media and those formulated and assessed in this study.

Callus growth on modified media

Four week-old callus (± 0.03 g, fresh mass) cultured on MS medium containing 30 g l^{-1} sucrose, 10 g l^{-1} agar and 3 mg l^{-1} 2,4-D (pH 5.6-5.8) was transferred onto media MM1, MM2, MM3 and MM4. These media all contained 30 g l^{-1} sucrose, 9 g l^{-1} Gelrite® (except 5 g l^{-1} in MM1) and 1 mg l^{-1} 2,4-D. The MM3 and MM4 nutrient media were compared to standard MS callus induction media at both pH 5.6 (MM1) and pH 4 (MM2) (Figure 3.5). The growth of callus was evaluated on these media without aluminium. The results show that calli growing on medium MM4 after 6 weeks had significantly higher growth rates than calli on the other treatments, including medium MM1 (standard MS at pH 5.6). Medium MM4 was, therefore, selected to screen *C. dactylon* callus for Al^{3+} resistance.

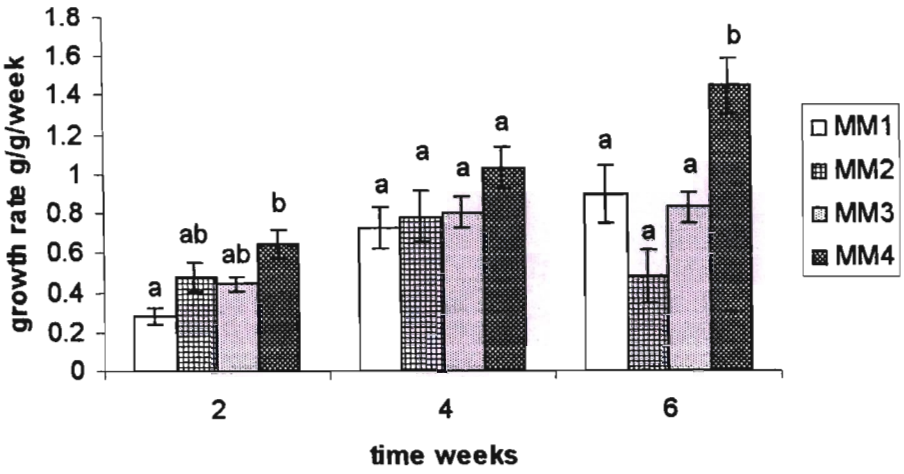

Figure 3.5 The effect of modified culture media on callus growth rate of *C. dactylon*. All callus induction media included 30 gl^{-1} sucrose, 9 gl^{-1} Gelrite® (except 5 gl^{-1} in MM1) and 1 mg l^{-1} 2,4-D. The media were: (MM1) MS standard, pH 5.6; (MM2) MS standard, pH 4; (MM3) MS modified (0.1 mM $CaCl_2$, 0.01 mM KH_2PO_4, 0 mM EDTA), pH 4; (MM4) MS modified (1 mM $MgSO_4$, no EDTA), pH 4. The mean growth rate for each medium type was compared at each time interval using the Scheffé's multiple range test ($p < 0.05$, n=20). Treatments with the same letter are not significantly different.

Al^{3+} screening medium

The catechol violet colorimetric method was also used to evaluate MINTEQA2 predictions of free Al^{3+} ion availability in the modified MM4 medium (Table 3.4). Medium MM4 contained MS nutrients modified by the addition of 1 mM $MgSO_4$. $7H_2O$, no EDTA, 30 g l^{-1} sucrose and 1 mg l^{-1} 2,4-D, and set at pH 4 (before autoclaving). The aluminium stock solution (25 mM $AlCl_3$) was set at pH 4 and added to medium MM4 after autoclaving to yield concentrations of 1, 4, 6 and 10 mM $AlCl_3$. Medium MM4 that included 0, 1 and 4 mM $AlCl_3$ also contained 9 g l^{-1} Gelrite®, whereas 13 g l^{-1} Gelrite® were used for media with 6 and 10 mM $AlCl_3$. This modified medium was set at pH 4. The pH of medium MM4 after autoclaving and the addition of $AlCl_3$ was as follows: media with 0 and 1 mM $AlCl_3$, pH 4 and media with 4, 6 and 10 mM $AlCl_3$, pH 3.6. The pH of the $AlCl_3$ stock solution decreased to 3.5 after autoclaving, thus reducing the pH of media with high $AlCl_3$ concentrations (i.e. 4, 6 and 10 mM) from 4 to 3.6. Therefore, to ensure that all the treatments were at the same pH, the pH of the $AlCl_3$ stock solution and that of the MM4

medium was set at 3.6 such that the final pH of all media with 0, 1, 4, 6 and 10 mM $AlCl_3$ was at 3.6.

Table 3.4 An assessment of MINTEQA2 predictions of Al^{3+} availability in the screening medium

Treatment mM $AlCl_3$	Predicted mM Al^{3+}	Assayed mM Al^{3+}
0	0	0.0016 ± 0.001
1	0.08	0.076 ± 0.01
4	0.83	0.82 ± 0.03
6	1.79	1.91 ± 0.018
10	1.91	2.28 ± 0.024

MINTEQA2 was run at pH 3.6 and precipitation was allowed. Catechol violet colorimetric assay was used to measure Al^{3+}. Column 3 shows the mean assayed value, ± SE, n = 12.

The free Al^{3+} ions predicted in MINTEQA2 simulations of medium MM4 at pH 3.6 with 0, 1, 4, 6 and 10 mM $AlCl_3$, compares favourably with those obtained through colorimteric analyses (Table 3.4). In subsequent investigations the aluminium content of the culture medium is quoted as 0, 0.08, 0.8, 1.9 and 2.3 mM Al^{3+} at pH 3.6.

3.2.5 Growth measurements

Approximately 0.03 g fresh mass of four-week-old calli from each genotype were weighed under sterile conditions and transferred onto medium MM4 containing 0 (control), 0.08, 0.8, 1.9 and 2.3 mM Al^{3+}. These media also contained 30 g l^{-1} sucrose, 9-13 g l^{-1} Gelrite® and 1 mg l^{-1} 2,4-D set at pH 3.6. Media (20 ml) were dispensed into 65 mm Petri dishes. Calli were weighed and transferred onto fresh media at 2 weekly intervals for a total of 8 weeks. Calli from the three *C. dactylon* genotypes cultured on medium MM4 were maintained in the dark (25 °C day/18 °C night) for a total of 8 weeks. The fresh mass (fm in g) of calli were recorded at two weekly intervals for a total period of 8 weeks when it was

used to calculate growth rate (g g^{-1} w^{-1}; e.g. for growth rate from week 0 to week 2: (($fm_{week\ 2} - fm_{week\ 0}$ / $fm_{week\ 0}$) / 2). Each treatment contained 20 replicates and these were scored for being either embryogenic or non-embryogenic. A replicate callus clump was scored as being embryogenic when 50% or more of it appeared compact, opaque and white to pale yellow in colour. The embryogenic calli contained small cells with a prominent nucleus whereas large tubular shaped (often enucleated) cells characterised the non-embryogenic callus (Figure 2.3). The number of non-embryogenic calli replicates, which appeared soft, gelatinous and translucent, in each treatment were expressed as a percentage of the total number of replicates. Thus, the embryogenic or non-embryogenic percentages are based on frequency measurements.

3.3 Results

3.3.1 Relationship between Al^{3+} concentration, culture time and genotype

A multifactorial ANOVA was performed on the transformed (square root) callus growth rate data (Table 3.5). This data set was transformed because it was significantly different from the normal distribution and ANOVA is reliant upon a parametric data set. These analyses examined each independent factor (genotype, Al^{3+} concentration and time) with regard to the dependent variable (callus growth rate) and considered the interactions among the three factors. There is a significant effect of each independent factor on callus growth rate (Table 3.5). These analyses indicate that all three genotypes are significantly different from each other. Further, the mean callus growth rate obtained for each Al^{3+} concentration is significantly different from each other, with growth rate values decreasing with increasing Al^{3+} concentration. There were no significant differences between the mean growth rate values calculated at 2 and 4 weeks ($p < 0.52$) and between 6 and 8 weeks ($p < 0.307$).

Table 3.5 A summary of results from the multifactor ANOVA examining effects of genotype, Al^{3+} concentration and culture time on callus growth rate

Source of variation	DF	Sum of Squares	Mean square	F	Significance p
Corrected model	59	39.150*	0.664	35.452	< 0.001
Intercept	1	461.895	461.895	24667.7	< 0.001
Three factors:					
Genotype	2	7.121	3.561	190.237	< 0.001
Treatment	4	22.93	5.732	306.266	< 0.001
Time	3	1.983	0.661	35.318	< 0.001
Two-way interactions:					
Genotype x Treatment	8	2.004	0.251	13.384	< 0.001
Genotype x Time	6	1.164	0.194	10.364	< 0.001
Treatment x Time	12	3.341	0.278	14.875	< 0.001
Three-way interaction:					
Genotype x Treatment x Time	24	0.778	3.24×10^{-2}	1.732	< 0.016
Error	1132	21.188	1.87×10^{-2}		
Total	1192	524.006			
Corrected total	1191	60.338			

* $r^2 = 0.649$ (adjusted $r^2 = 0.631$). Analyses performed on transformed (square root) callus growth rate data.

These analyses further indicate that there were significant interactions among the three factors investigated. Increasing Al^{3+} concentration had a greater negative effect on callus growth rate of genotype 2 than genotypes 1 and 3 (Figure 3.6). In addition, the confidence limits for the mean callus growth rate calculated (in the absence of Al^{3+}) for each of the

three genotypes overlapped, indicating that there were no significant differences among the three genotypes for all tested culture intervals. Other interactions showed that the change in callus growth rate over time differed among the three genotypes and the negative effects of increasing Al^{3+} concentration on callus growth rate appeared more distinct with increased culture time than after a short culture interval.

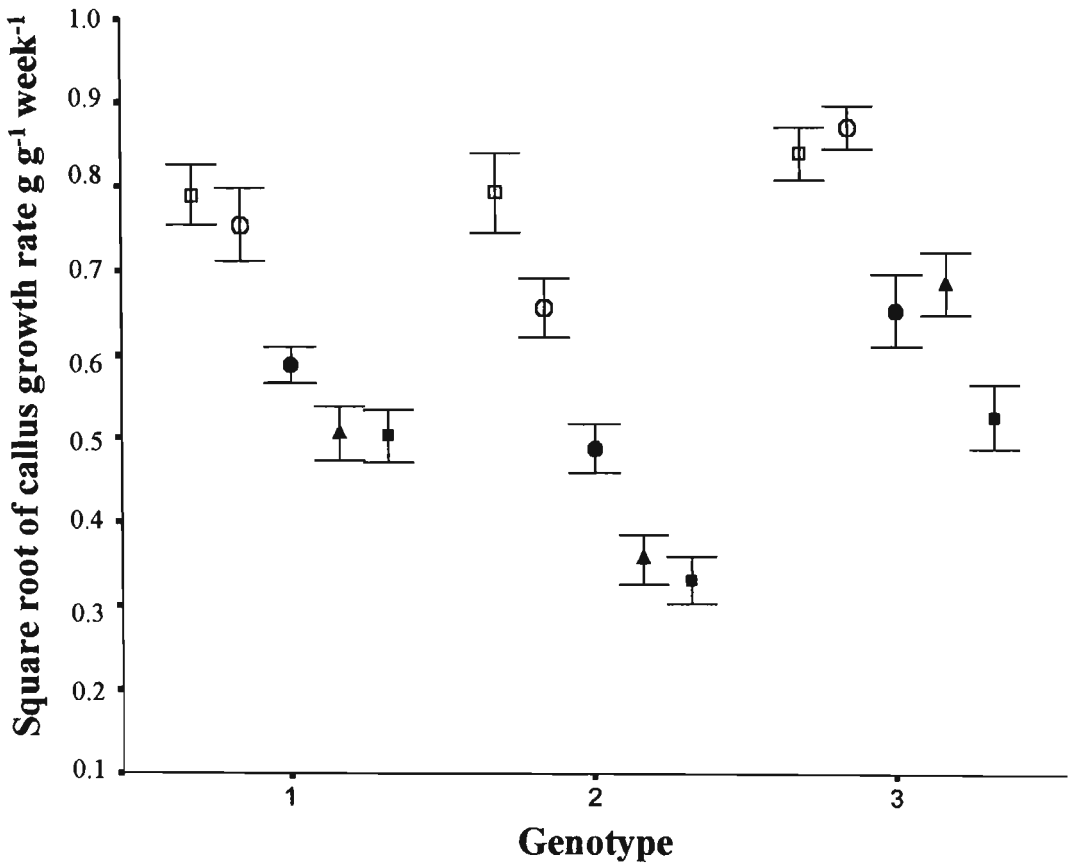

Figure 3.6 The influence of genotype and Al^{3+} concentration on callus growth rate. Al^{3+} concentrations: 0 □, 0.08 ○, 0.8 ●, 1.9 ▲ and ■ 2.3 mM Al^{3+}. Genotype 1 = moderately Al^{3+}-resistant, Genotype 2 = Al^{3+}-sensitive, Genotype 3 = Al^{3+}-resistant. The mean value (± 95% confidence limit) are represented in the graphs and compared using a multifactor ANOVA, $p < 0.05$.

3.3.2 Response of each genotype to increasing Al^{3+} concentration at each culture interval

Callus growth

A One-Way Analysis of Variance was used to compare the response of each genotype, at each specified time interval, to Al^{3+} (Figure 3.7). Upon exposure to Al^{3+} the response of each of the three genotypes was different from each other. The growth rate of genotype 1 callus was significantly lower than the control at 2 weeks for 1.9 mM Al^{3+} and at 4, 6 and 8 weeks at Al^{3+} concentrations of 0.8, 1.9 and 2.3 mM (Figure 3.7). It was evident that the growth rate of callus exposed to 0.8, 1.9 and 2.3 mM Al^{3+} were all significantly lower than the control, but not significantly different from each other. Also at each culture interval there was no significant difference in the growth rate of callus exposed to 0.08 mM Al^{3+} when compared to the control.

In genotype 2 at all tested time intervals, except 4 weeks exposure to 0.08 mM Al^{3+} resulted in a significantly lower growth rate than the control. Further, similar to genotype 1, there were no significant differences in the growth rate of callus exposed to 0.8, 1.9 and 2.3 mM Al^{3+} at each time interval.

In genotype 3, after 2 weeks of culture, only the highest Al^{3+} concentration tested (2.3 mM) resulted in a significant reduction in callus growth rate when compared to the control. As in genotype 1, the lowest Al^{3+} concentration had no effect on callus growth rate at all tested time intervals. Only after 6 and 8 weeks of culture was the growth rate of callus exposed to 0.8, 1.9 and 2.3 mM Al^{3+} significantly lower than the control.

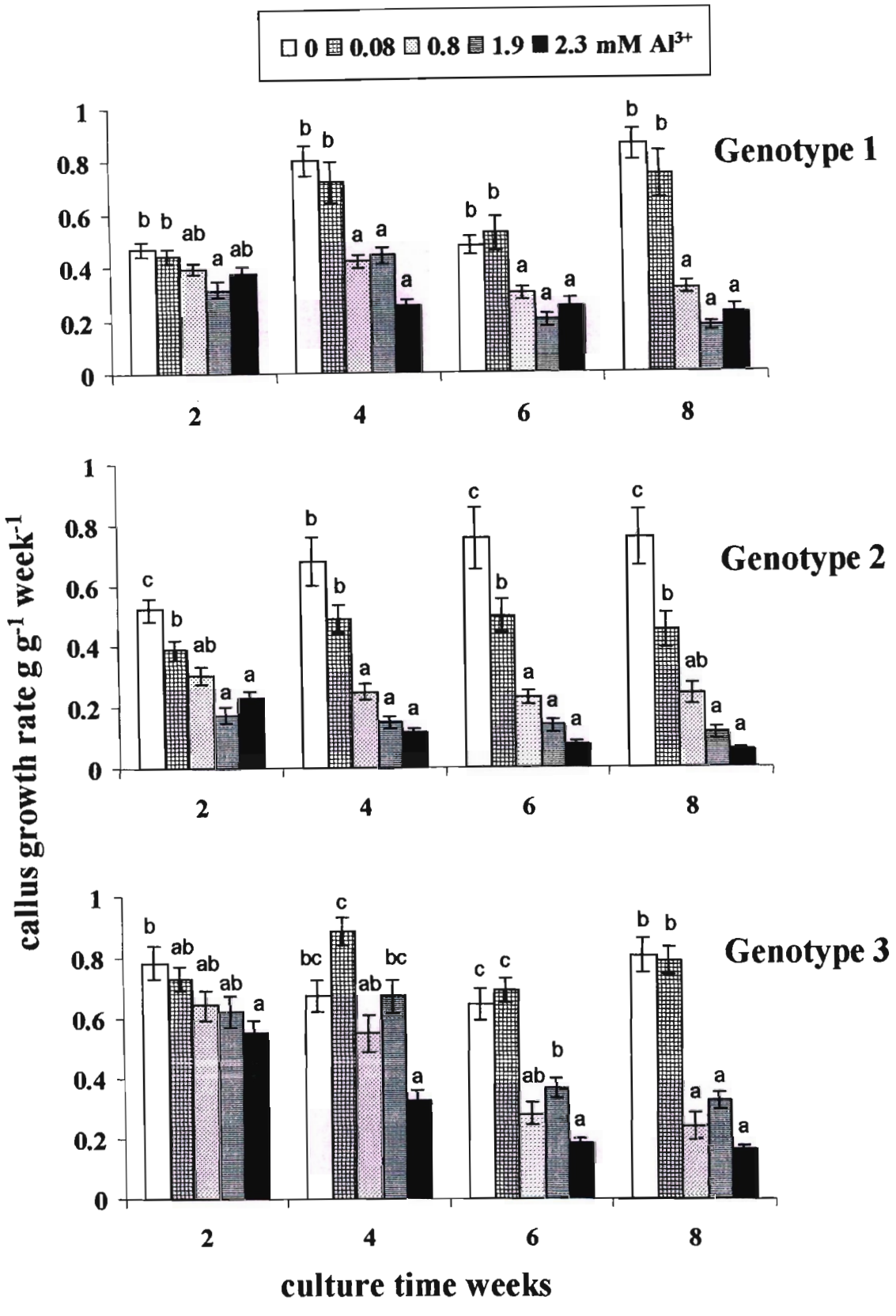

Figure 3.7 The influence of increasing Al^{3+} concentration and culture time on callus growth rate of three *C. dactylon* genotypes. Genotype 1 = moderately Al^{3+}-resistant, Genotype 2 = Al^{3+}-sensitive, Genotype 3 = Al^{3+}-resistant. The means were compared at each time interval for each genotype using a One-way ANOVA and the Scheffe's multiple comaprison test ($p < 0.05$, $n = 20$). Dissimilar alphabet characters denote a statistical significance. Histograms represent the mean growth value for each treatment (± SE).

Frequency of non-embryogenic callus

In addition to the growth rate, the type of callus formed in response to Al^{3+} was also recorded (Figure 3.8). In response to Al^{3+} exposure, embryogenic callus with normal meristematic cells were found to convert, in part, into non-embryogenic callus containing irregular cells. In this regard, both Al^{3+} concentration and exposure time had an effect on the production of non-embryogenic callus in all three *C. dactylon* genotypes (Figure 3.8). In genotype 1, non-embryogenic callus was not observed in the control in the first 6 weeks of culture. At the lowest Al^{3+} concentration (0.08 mM) approximately 20% of the cultures had formed non-embryogenic callus in weeks 4, 6 and 8. Further, more than 60% of cultures exposed to 1.9 and 2.3 mM Al^{3+} had formed non-meristematic callus at all the culture intervals. In contrast to genotype 1, at least 20% of genotype 2 control callus was non-embryogenic in weeks 4, 6 and 8. Exposure of embryogenic callus to 2.3 mM Al^{3+} resulted in the conversion of more than 80% of the cultures into a non-embryogenic state, after 2 weeks. Non-embryogenic callus was only induced in genotype 3 after 2 weeks of culture at 1.9 and 2.3 mM Al^{3+}. Similar to genotype 1, non-embryogenic callus was not observed in the control cultures in the first 6 weeks of culture.

In all three genotypes it appeared that at each culture time increasing Al^{3+} concentration resulted in increased non-embryogenic callus formation. Further, it seemed that exposure to Al^{3+} resulted in the conversion (into a non-embryogenic state) of a greater proportion of genotype 1 and genotype 2 embryogenic cultures than genotype 3. For example at 4 weeks, 90% of genotype 1 and genotype 2 cultures exposed to 2.3 mM Al^{3+} were non-embryogenic while less than 40% of genotype 3 cultures were in the same non-embryogenic state.

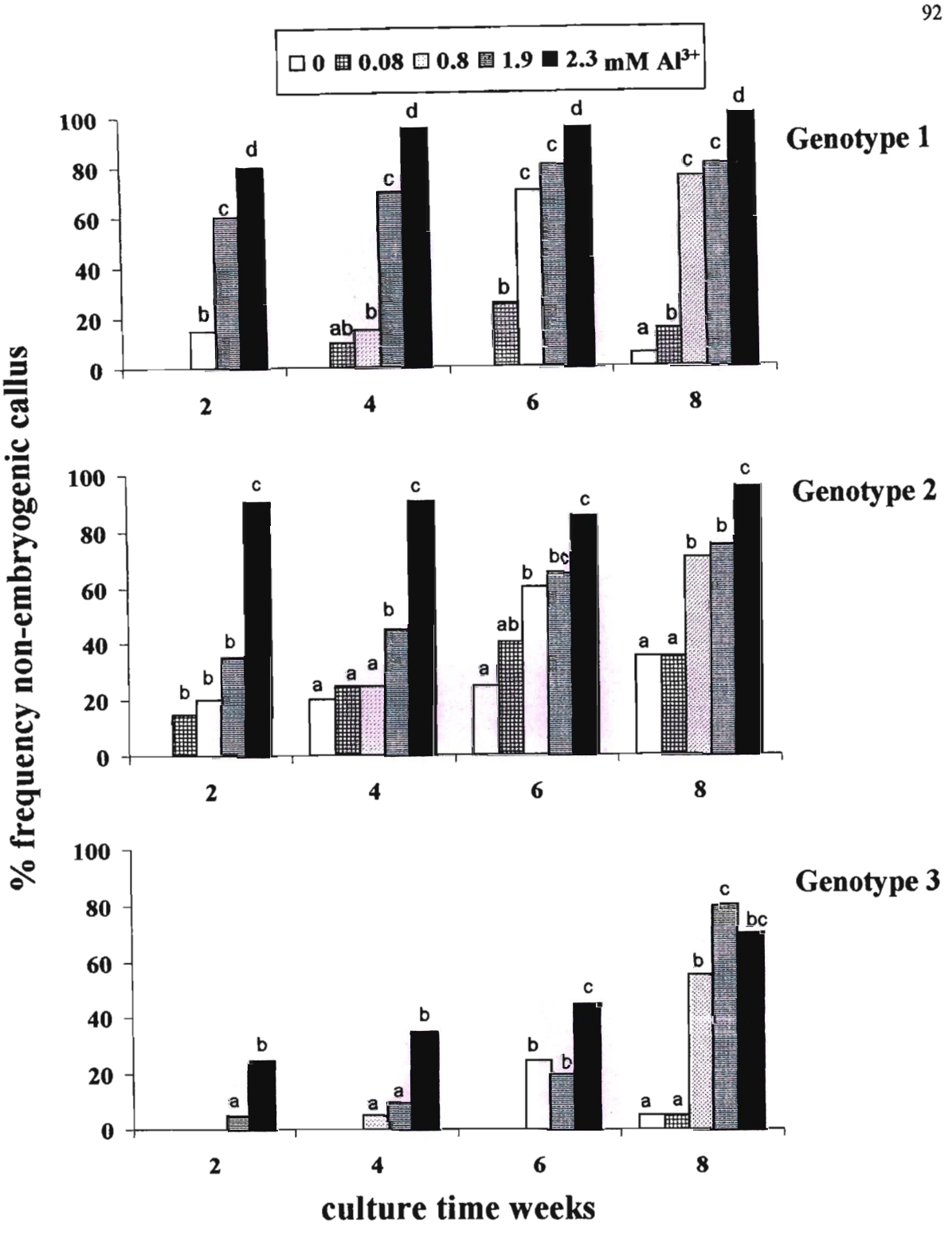

Figure 3.8 The effect of increasing Al^{3+} concentration and culture time on the frequency of non-embryogenic callus production. Genotype 1 = moderately Al^{3+}-resistant, Genotype 2 = Al^{3+}-sensitive, Genotype 3 = Al^{3+}-resistant. The means were compared at each time interval for each genotype using a Chi-squared test ($p < 0.05$, $n = 20$). Dissimilar alphabet characters denote a statistical significance.

3.3.3 Al^{3+}-resistance rating

Multifactor ANOVA

The mean response of each genotype was calculated for all tested Al^{3+} concentrations (0-2.3 mM) and time intervals (2-8 weeks) and compared using a Scheffé's test. The transformed data (square root) were used and the mean values were back-transformed to mean callus growth rate for each genotype and presented in Table 3.6. The mean value for each genotype was significantly different from each other ($p < 0.001$), with genotype 2 having the lowest growth value (0.278 g g^{-1} $week^{-1}$) and genotype 3 the highest (0.513 g g^{-1} $week^{-1}$). This statistical analysis identified genotype 2 as Al^{3+}-sensitive, genotype 1 as moderately Al^{3+}-resistant and genotype 3 as Al^{3+}-resistant.

Table 3.6 A comparative response of the three genotypes calculated for all tested Al^{3+} concentrations and time intervals

Genotype	n	Mean growth rate g g^{-1} $week^{-1}$		
		I	II	III
2	397	0.278 a		
1	400		0.396 b	
3	395			0.513 c

A multifactor ANOVA and a Scheffé's multiple comparison test was applied to the mean growth rate for the three genotypes, calculated for all time intervals (weeks 2-8) and Al^{3+} concentrations (0-2.3 mM). Dissimilar alphabet characters represent a statistical significance, $p < 0.001$.

Two-way ANOVA

To determine the shortest time and lowest Al^{3+} concentration for screening *C. dactylon* genotypes for Al-resistance, a Two-way ANOVA was performed on the 2-week data. The main effects of genotype and Al^{3+} concentration were significant (Genotype: $F = 105.1$, $p < 0.001$; Al^{3+} concentration: $F = 21.677$, $p < 0.001$), indicated previously with the multifactor (Three-way ANOVA) analysis. A Scheffé's multiple comparison test was used to determine the lowest Al^{3+} concentration that significantly reduced callus growth rate when compared to the control (0 mM Al^{3+}), this Al^{3+} concentration was 0.8 mM.

3.4 Discussion

Calli of three *C. dactylon* genotypes were exposed to Al^{3+} on a modified *in vitro* nutrient medium, to assess the feasibility of using differences in callus growth to identify potential Al-R individuals. Callus was initiated in the first stage of culture of the somatic embryogenesis protocol (section 2.3.2). That callus culture medium (MS, 30 g l^{-1} sucrose, 10 g l^{-1} agar and 3 mg l^{-1} 2,4-D, pH 5.6-5.8) was amended in order to ensure that a known supply of Al^{3+} would interact with the meristematic callus cells. Apart from the addition of an Al source (i.e. $AlCl_3$), the modifications involved amendments to the nutrient medium formulation and a reduction in pH.

A number of authors followed the suggestions of Conner and Meredith (1985ab) in order to increase free Al ion availability in the *in vitro* culture media (Parrot and Bouton, 1990; Yamamoto *et al.*, 1994; Espino *et al.*, 1998; Devi *et al.*, 2001). Those amendments included decreased pH, the removal of EDTA and reductions in levels of PO_4^{2-} and Ca^{2+}. In the present study, a speciation model (MINTEQA2) was used to assess the merits of those published Al screening media, including that of Conner and Meredith (1985a) (Figure 3.3). Only modifications that increased free Al^{3+} ion activity in the *C. dactylon* callus culture medium were implemented (Figure 3.4), and these were a decrease in medium pH (from 5.6 to 3.6), a reduction in $MgSO_4$ (from 1.7 to 1 mM) and the removal of EDTA. Although Conner and Meredith (1985a) reported that PO_4^{2-} and Ca^{2+} reduced Al availability in the culture media, in this work levels of these ions were not decreased since MINTEQA2 simulations showed that (at the levels present in the MS medium) they did not interact with Al^{3+}. Levels of $MgSO_4$ were reduced since the MINTEQA2 model predicted that increased SO_4^{2-} ions resulted in increased Al precipitation (alunite) in the callus culture medium. This component of the MS nutrient formulation was reduced since a decrease in Mg^{2+} did not have a negative effect on callus growth in the control medium (Figure 3.5).

In this work $AlCl_3$ was selected over $Al_2(SO_4)_3$ since both MINTEQA2 simulations (Figures 3.1 and 3.2) and quantitative measurements (Table 3.4) showed that $AlCl_3$ provided a greater supply of free Al^{3+} ions than $Al_2(SO_4)_3$. It was shown that $Al_2(SO_4)_3$ increased the SO_4^{2-} ions in the medium, resulting in increased Al precipitation (Figure 3.3).

Aluminium sulphate was used in early Al screening studies (Smith *et al.*, 1983; Conner and Meredith, 1985a; Campbell *et al.*, 1989) and its use still continues (Van Sint Jan *et al.*, 1997; Toan and Debergh, 2002). Predictions from the MINTEQA2 model indicated that free Al^{3+} ion activity decreased with increasing $Al_2(SO_4)_3$ supply in the culture media of Conner and Meredith (1985a), Campbell *et al.* (1989) and Van Sint Jan *et al.* (1997) (Figure 3.3). Therefore, the question arises whether the Al screening media in those published studies provided an available source of the phytotoxic Al^{3+} species since according to the speciation model those media support an increased supply of $AlSO_4^+$ and $AlSO_4^{2-}$ ions and not Al^{3+}. The $AlSO_4^+$ ion has been reported to be ten times less toxic than Al^{3+} (Kinraide and Parker, 1987a), with the Al^{3+} ion being considered as phytotoxic and responsible for root growth inhibition at low pH (Kinraide, 1991, 1997). Thus it is concluded here that in order to ensure free Al^{3+} ion availability in the callus medium $AlCl_3$ must be used as the Al source.

In the present investigation, the MINTEQA2 model was set to allow for precipitation when predicting Al^{3+} activity since preliminary studies showed that an Al precipitate was formed in the culture medium. A maximum Al^{3+} activity of 7.5 μM was predicted for medium MM4 (Figure 3.4) and other published *in vitro* screening media (Figure 3.3). In contrast authors of other studies using the GEOCHEM speciation model, reported higher Al^{3+} activities than those reported in this study for inhibition of root elongation ((42.5 μM) Rincon and Gonzales, 1992; (0.5-8 μM) Bianchi-Hall *et al.*, 1998; (10.3 μM) Pintro *et al.*, 1998; (2-450 μM) Villagarcia *et al.*, 2001). In those studies a maximum Al^{3+} activity was not reached and Al^{3+} activity was reported to increase with increasing Al supply (Crawford and Wilkens, 1998), since the GEOCHEM model was not set to allow for precipitation.

The MM4 Al screening medium was selected since it provided an increasing range of Al^{3+} activities when compared to other Al screening media (Figure 3.4) and in the absence of Al this callus culture medium had no negative effects on callus growth (Figure 3.5). In the present study, results from MINTEQA2 simulations indicated that when a maximum Al^{3+} activity of 7.5 μM was reached then further increases in $AlCl_3$ supply did not result in increasing Al^{3+} activity. This was true for medium MM4 (Figure 3.4) and other published

media (Figure 3.3). In medium MM4 this maximum activity was reached at 2 mM $AlCl_3$, therefore, since the influence of $AlCl_3$ concentrations greater than 2 mM were investigated on callus growth, the predicted Al^{3+} concentration and not activity was considered in subsequent investigations. The measured Al^{3+} ions in the MM4 medium compared favourably with those predicted by the MINTEQA2 model, suggesting that the model was accurately predicting the interactions of Al in the nutrient medium (Table 3.4). In all subsequent experiments medium MM4 (30 g l^{-1} sucrose, 9-13 g l^{-1} Gelrite®, 1 mg l^{-1} 2,4-D and 0, 0.08, 0.8, 1.9 and 2.3 mM Al^{3+}, at pH 3.6) was used to investigate the responses of *C. dactylon* callus to Al^{3+}.

When meristematic callus cells of three *C. dactylon* genotypes were exposed to five Al^{3+} concentrations (0-2.3 mM) and four culture intervals (2-8 w), statistical analyses indicated that there was a significant interaction among all three factors (Table 3.5). In the absence of Al^{3+}, there were no significant differences in the mean callus growth rate of the three genotypes, calculated for all tested time intervals (Figure 3.6). Also, all three genotypes maintained a high mean growth rate (0.64 g g^{-1} $week^{-1}$). This is essential for screening for Al-resistance since Yamamoto *et al.* (1994) reported that cells of *Nicotiana tabacum* in culture must be in an active growth phase to enable Al uptake and interaction.

Results of the present study showed that both Al^{3+} concentration and culture time influenced callus growth rate in all three genotypes. Also, the response of the three genotypes to Al^{3+} was significantly different from each other (Table 3.5, Figure 3.6). Statistical analyses of the callus growth rate indicated that genotype 2 was Al^{3+}-sensitive and genotype 3 Al^{3+}-resistant. Thus suggesting that differences in callus growth rate (when exposed to Al^{3+}) could be used to differentiate between an Al-S and Al-R genotype. In other published studies, Al-R individuals are usually selected through the use of the lowest Al concentration and the shortest culture time that significantly reduces growth when compared to the control or reduces growth by 50%. For example, the growth of *Coffea arabica* cells was reduced by 50% when exposed to 25 µM $AlCl_3$ for 14 d (Martínez-Estévez *et al.*, 2001) and *Glycine max* root growth was reduced significantly in the presence of 50 µM $AlCl_3$ for 1 h when compared to the control (Kataoka and Nakanishi, 2001). In

this study a culture regime of 0.8 mM Al^{3+} and 2 weeks could be used to identify an Al-R *C. dactylon* genotype.

In other callus culture studies, longer exposure periods than those used in the present work were required when screening for Al-resistance, for example: 8 weeks (0.4 mM $AlCl_3$) for *Medicago sativa* (Parrot and Bouton, 1990) and 4 weeks (2.08 mM $AlCl_3$) for *Phaseolus vulgaris* (Espino *et al.*, 1998). In those studies, the long culture time could be attributed to a low availability of free Al^{3+} ions resulting from interactions of Al with components in the culture medium. Although media used by the authors in those studies were modified, using the suggestions of Conner and Meredith (1985ab), other components (discussed earlier) could also reduce Al levels thus increasing screening time. Reductions in the levels of Ca^{2+} and PO_4^{2-} in those screening media and extended culture intervals could result in a nutrient stress. Thus in the development of Al screening media for callus cultures it is essential to ensure both an available supply of free Al^{3+} as well as adequate nutrient levels for active callus growth.

Some authors, using the conventional approach to screening for Al-resistance, simplified the screening medium to a low strength single salt solution in order to minimise Al interactions with components of the nutrient screening medium and maximise Al^{3+} availability (Bianchi-Hall *et al.*, 1998; Kataoka and Nakanishi, 2001; Villagarcia *et al.*, 2001 and Vasconcelos *et al.*, 2002). Such single salt solutions have been also adopted by researchers using *in vitro* techniques to select Al-resistant individuals. In some recent studies media for cell suspension cultures were also modified to a simple salt solution (Ikegawa *et al.*, 2000; Schmohl and Horst, 2002; Zhu *et al.*, 2003). This approach is possible since culture times for screening cell suspensions for Al-resistance are short (2-24 h) and a short exposure time will therefore minimise nutrient stress. A simple salt solution is not a feasible Al screening medium for callus cultures since they require longer exposure times than cell suspensions and this would result in nutrient deficiencies.

In the present study it was also possible to differentiate among the three genotypes using the frequency of non-embryogenic callus production (Figure 3.9). Although these data are

qualitative (derived through visual assessment), the method may be useful for a preliminary selection process, especially since it is non-destructive. At 0.8 mM Al^{3+} and 2 weeks, 20% of the cultures of genotype 1 and 2 were non-embryogenic, whereas genotype 3 showed no non-embryogenic callus (Figure 3.9). The frequency of non-embryogenic callus production in response to Al^{3+} may be regarded as a simple measure similar to the hematoxylin staining of roots (Ownby, 1993; Bennet, 1995). Thus an assessment of the frequency of non-embryogenic callus may be a useful preliminary measure of Al-resistance.

A limitation to this alternative (*in vitro*) approach to investigating Al-resistance is the potential loss of resistance through somaclonal variation (Joyce *et al.*, 2003). Actively growing callus is under a state of rapid cell division and genetic mutations may result during this process. Some researchers induce this event through the exposure of callus to Al for long culture times in order to regenerate Al-resistant genotypes. In such a study Van Sint Jan *et al.* (1997) induced Al-R individuals from a sensitive rice cultivar after 20 weeks in culture. However, those mutations may also result in the opposite effect with the loss of Al resistance, for example as shown by Moon *et al.* (1997) with the production of an Al-S genotype from an Al resistant maize plant. In the present study somaclonal variation was minimised by maintaining a short culture period (2 weeks), using newly initiated (young, 4 week-old) callus and a low 2,4-D supply. Another possible disadavantage of this *in vitro* approach is that the pH of the Al screening culture medium is low (< 4 to ensure maximum free Al^{3+} ions) and most cell culture systems are at pH 5.6-5.8. It is possible that the *in vitro* response of some species (i.e. callus growth) could be negatively affected by the low pH in the absence of Al. This did not appear to be the case in the present study since the control callus (-Al, pH 3.6) maintained a high growth rate for the total culture time investigated (Figure 3.6).

In this study, genotype 2 was identified as Al^{3+}-S, genotype 1 as moderately Al^{3+}-R and genotype 3 as Al^{3+}-R (Table 3.6). The three *C. dactylon* genotypes were also assessed for resistance to Al^{3+} using the conventional approach (root growth in hydroponics) by other researchers in our laboratory (unpublished data). In those studies, using root growth measurements, the three genotypes were ranked in order of increasing resistance to Al:

genotype 2, genotype 1 and genotype 3. It appears, therefore, that with regard to the identification of Al-R individuals, the results obtained using the *in vitro* approach with callus cells compare favourably with those obtained conventionally with whole plants.

Clearly, a point of difference between the root growth and *in vitro* cell culture approach is that the former screens at the whole plant level and the latter at the cellular level. For the development of a suitable *in vitro* Al^{3+} screening system it is important that Al resistance is expressed at the cellular level. A number of previous studies have been discussed to show the feasibility of screening for Al^{3+} resistance at the cellular level, using both cell suspension (Ojima and Ohira, 1983; Conner and Meredith, 1985a, Yamamoto *et al.*, 1994; Koyama *et al.*, 1995; Ikegawa *et al.*, 1998) and callus (Campbell *et al.*, 1989; Parrot and Bouton, 1990; Van Sint Jan *et al.*, 1997; Espino *et al.*, 1998) cultures. In an important review, Taylor (1995) concluded that resistance to Al is expressed both at the whole plant level as well as in undifferentiated meristematic cells of callus and suspension cultures and that this resistance expressed at the cellular level is stable and allows for the regeneration of Al resistant plants. In the present work it was possible using differences in callus growth to differentiate between an Al-S and Al-R *C. dactylon* genotype. An advantage of this approach is being able to facilitate investigations into Al^{3+} toxicity and resistance at the cellular level. This aspect is investigated in the next chapter.

CHAPTER 4: RESPONSES OF Al-SENSITIVE AND Al-RESISTANT MERISTEMATIC CALLUS CELLS TO Al^{3+}

4.1 Introduction

As discussed earlier (chapter 1), a rapid inhibition of root elongation is a widely accepted measure of Al stress in plants (Kochian, 1995; Matsumoto, 2000). Root growth is restricted since Al interferes with the functioning of cells in the mitotic and elongation stages in the root tip (Kochian, 1995, Čiamporová, 2002). The root apical meristem comprises a group of actively dividing cells that is followed by a region of elongation that allows the root to grow in the direction of the root axis. Meristematic cells undergoing a preparatory phase prior to elongation (DTZ) are most sensitive to Al (Sivaguru and Horst, 1998). Both meristematic and elongating cells are in an indeterminate state (Nobel, 1999). In this study, callus (an undifferentiated mass of loosely arranged actively dividing cells), was used to study the effects of Al^{3+} on two *C. dactylon* genotypes.

The objective of the present study was to investigate the effects of Al^{3+} on Al-sensitive and Al-resistant meristematic callus cells. These cells resulting from Al-S and Al-R genotypes were exposed to Al^{3+} and their responses to Al^{3+} during cell division were studied. A meristematic callus culture system has been developed in order to screen *C. dactylon* genotypes for resistance to Al^{3+} (see chapter 3). Differences in callus growth rates in the presence of Al^{3+} have been used to select an Al-S and an Al-R genotype (Genotype 2 and 3, see chapter 3). In the present chapter results of cellular investigations are reported using these two genotypes in order to ascertain reasons for their different responses to Al^{3+}.

As mentioned in the general introduction (chapter 1), Al appears to target many critical cellular functions in the root tip, resulting in inhibited root growth (Matsumoto, 2000; Čiamporová, 2002). Of those the Al-induced interference in the process of cell division has received the most interest (Kochian, 1995; Matsumoto *et al.*, 2001; Čiamporová, 2002). Therefore, in this study the influence of Al^{3+} on the rapidly dividing meristematic callus cells was assessed. It has been suggested in the literature that Al can inhibit mitosis through a possible interference in DNA synthesis (Wallace and Anderson, 1984) or through a

disruption in the cytoskeleton of the cell (Sivaguru *et al.*, 1999b). Therefore the location of Al within the cell could provide vital information as to the mechanism of Al toxicity. The aluminium content of Al-S and Al-R cells was measured in order to determine the intracellular targets of Al^{3+}. In addition, any differences in the amount of Al detected within the callus cells could also be used to determine whether the Al-R genotype adopts possible Al^{3+} exclusion strategies. Many researchers support the idea that Al-resistance is linked with Al avoidance such that Al-R genotypes are able to exclude Al and prevent the cells from injury. In contrast, Al-S genotypes are thought to have inefficient or poorly developed Al exclusion systems such that Al accumulates in cells and causes cellular damage (Kochian, 1995; Matsumoto *et al.*, 2001). Aluminium exclusion strategies proposed for roots of whole plants have also been investigated in the present study.

Various methods have been established to locate and measure Al in plant cells and examples of those methods are presented in Table 4.1. Some methods are qualitative and offer a simple and rapid means of locating accumulated Al in roots or plant cells. One such method involves the use of the Al stain hematoxylin that forms a blue-purple colour when complexed with Al (e.g. Rincon and Gonzales, 1992). As discussed previously (section 3.1), the hematoxylin method of determining Al accumulation in roots was developed to screen for Al tolerance in several wheat varieties (Polle *et al.*, 1978). Aluminium was shown to accumulate mainly in the apical region of wheat roots, with the Al-S roots staining more intensely than those of the Al-R. Those results suggested that the Al-S genotype contained more Al in the root tissue than the Al-R and this staining technique was used as a rapid means of identifying Al-R individuals (Rincon and Gonzales, 1992; Delhaize *et al.*, 1993a; Bona and Carver, 1998). The hematoxylin method has also been used to localise Al in cells of callus cultures. For example, meristematic *Phaseolus vulgaris* callus cells exposed to Al showed an intense blue colour when stained with hematoxylin whereas the control calli stained brown (Espino *et al.*, 1998). The hematoxylin staining technique is a rapid and simple method for the visual detection of Al in plant cells and its use continues in spite of the development of more sensitive and accurate Al detection techniques (Crawford *et al.*, 1998; Vázquez *et al.*, 1999; Yoshida and Yoshida, 2000; Table 4.1).

Table 4.1 Methods used to locate and quantify Al in plant cells

Method of detection	Qualitative/ Quantitative	Species	Plant material	Reference
Al stain:				
Hematoxylin	Qualitative	*Triticum aestivum*	root	Rincon and Gonzales, 1992
		Phaseolus vulgaris	callus	Espino *et al.*, 1998
		Zea mays	root	Vázquez *et al.*, 1999
Fluorescent Al indicator:				
Lumogallion	Qualitative	*Glycine max*	root	Kataoka *et al.*, 1997
Morin	Qualitative/ Quantitative	*Triticum aestivum*	root	Brauer, 2001
		Nicotiana tabacum	cell suspension	Vitorello and Haug, 1996
		Coffea arabica	cell suspension	Martínez-Estévez *et al.*, 2001
Spectrometry:				
aluminon*	Quantitative	*Solanum tuberosum*	cell suspension	Wersuhn *et al.*, 1994
pyrocatechol violet*	Quantitative	*Oryza sativa*	cell suspension	Rahman *et al.*, 1999

Table 4.1 contd.

Method of detection	Qualitative/ Quantitative	Species	Plant material	Reference
Spectrometry:				
GFAAS	Quantitative	*Zea mays*	root	Sivaguru and Horst, 1998
		Nicotiana tabacum	cell suspension	Yamamoto *et al.*, 1994
		Hordeum vulgare	cell suspension	Zhu *et al.*, 2003
X-ray microanalysis:				
EDXM-SEM	Qualitative/ Quantitative	*Avena sativa*	root	Marienfeld and Stelzer, 1993
EDXM-STEM	Quantitative	*Danthonia linkii* *Microlaena stipoides*	root	Crawford *et al.*, 1998
EDXM-TEM	Quantitative	*Zea mays*	root	Vazquez *et al.*, 1999
		Picea rubens	cell suspension	Minocha *et al.*, 2001

* Colorimetric Al indicators, GFAAS- Graphite Furnace Atomic Absorption Spectrometry, EDXM– Energy Dispersive X-ray Microanalysis, SEM- Scanning Electron Microscope, STEM- Scanning Transmission Electron Microscope, TEM- Transmission Electron Microscope.

Methods have been developed using fluorescent Al indicators (morin and lumogallion) that are sufficiently sensitive to detect small amounts of Al in plant cells (Tice *et al.*, 1992; Vitorello and Haug, 1996; Kataoka *et al.*, 1997). Morin shows a high specificity for Al and the Al-morin complex fluoresces green (Vitorello and Haug, 1996; Brauer, 2001; Martínez-

Estévez *et al.*, 2001). Morin fluorescence studies in *Nicotiana tabacum* cell cultures confirmed that Al uptake was critically dependent on cell growth (Vitorello and Haug, 1996). Later studies also linked inhibited cell division in *Coffea arabica* with increased Al uptake (Martínez-Estévez *et al.*, 2001).

Although fluorescent Al indicators were initially used as a qualitative measure of Al distribution in plant cells, some authors were able to quantify the accumulated Al by measuring the intensity of the fluorescence using a fluorospectrometer (Vitorello and Haug, 1996; Martínez-Estévez *et al.*, 2001; Brauer, 2001). In fact Vitorello and Haug (1996) compared this method for determining Al content of plant cells with graphite furnace atomic absorption spectrometry (GFAAS) and found that both methods yielded similar results. However, both atomic absorption and colorimetric Al analysis methods are reliant on the acid digestion of root tips or cell cultures (Wersuhn *et al.*, 1994; Sivaguru and Horst, 1998; Rahman *et al.*, 1999). This therefore means that the total Al content of root tissue or plant cells is determined. The fluorescence method offers the advantage of Al quantification and distribution within plant cells.

Another method that offers the same advantage of understanding intracellular Al distribution is energy dispersive X-ray microanalysis (EDXM) linked to scanning or transmission electron microscopy (SEM, TEM). This method for determining Al is based on the fact that electrons that collide with plant cells generate X-rays which are unique for each element detected. Detectors in the microscope are able to identify and quantify the characteristic X-ray spectra (Goldstein *et al.*, 1992). Samples for analysis are either frozen, cryo-sectioned and viewed with the SEM (Marienfeld and Stelzer, 1993) or frozen and chemically treated (freeze-substitution), sectioned and viewed with the TEM (Crawford *et al.*, 1998; Vazquez *et al.*, 1999; Minocha *et al.*, 2001). Thus this useful approach allows the identification of Al-induced ultrastructural alterations and provides Al and other elemental distribution patterns at the cellular level (Marienfeld *et al.*, 1995; Godbold and Jentschke, 1998).

The root apex has been identified as the primary site for Al accumulation and Al-induced injury causing inhibited root elongation (Kochain, 1995; Delhaize and Ryan, 1995; Matsumoto *et al.*, 2001; Čiamporová, 2002). A number of investigations on the spatial sensitivity of roots to Al have been conducted using *Zea mays*. In earlier studies, Bennet and Breen (1991) attributed the root cap to play a major role in the perception of Al toxicity in maize. However, Ryan *et al.* (1993) later showed that the meristematic zone (0-3 mm behind root cap) and not the root cap was the most Al-sensitive site in maize. Those authors also reported that the application of Al to mature regions of the root had no inhibitory effects on root growth. Sivaguru and Horst (1998) later divided the root apex of maize into meristematic (0-1 mm), transition (1-4 mm) and elongation (4-10 mm) zones and exposed them individually to Al. The transition zone was characterised by cells that were changing their mitotic state and undergoing a preparatory phase for rapid elongation. They reported that the distal part of the transition zone (DTZ, 1-2 mm) of maize roots was the most Al-sensitive site, followed by the meristematic zone (0-1 mm) and proximal part of the transition zone (2-3 mm). Application of Al to the DTZ resulted in the most severe inhibition in root elongation and maximum callose induction when compared to the other zones. It also appeared that Al accumulated preferentially in the DTZ.

Aluminium has been detected in nuclei of root apex cells after 15 min of exposure to Al (Kataoka and Nakanishi, 2001). Further, low amounts of Al in the nucleus appeared to have no inhibitory effects on root elongation. It seemed that a critical Al concentration was required in the nucleus to disrupt cell division and inhibit root growth (Kataoka and Nakanishi, 2001; Silva *et al.*, 2000). Some studies showed that Al accumulated preferentially in symplasmic sites of root apex cells (Crawford *et al.*, 1998; Kataoka and Nakanishi, 2001; Silva *et al.*, 2000) whereas others found Al localised predominantly in the apoplast (Marienfeld *et al.*, 1995; Godbold and Jentschke, 1998). However, regardless of whether Al accumulated in the apoplast or symplast there appears to be mounting evidence to suggest that inhibited root growth occurred as a result of Al interactions with cells of the root apex.

4.2 Materials and Methods

4.2.1 Plant material and callus induction

Callus was initiated from leaf bases of an Al-S and an Al-R *C. dactylon* genotype, referred previously (section 3.2.1) as Genotype 2 and 3, respectively. Root growth analyses in nutrient solution containing Al (performed by other researchers in this laboratory) confirmed the Al-S and Al-R rating of these two genotypes. The callus induction medium, established in the somatic embryogenesis protocol (section 2.2.3), includes MS nutrients, 30 g l^{-1} sucrose, 10 g l^{-1} agar and 3 mg l^{-1} 2,4-D at pH 5.6-5.8. Callus cultures were maintained in the dark (25 °C day/18 °C night) for 4 weeks, with a subculture onto fresh nutrient media after 2 weeks.

4.2.2 Al^{3+} and colchicine treatments

Four-week-old callus (± 0.03 g fresh mass) was transferred onto the Al screening medium: Medium MM4 (MS modified: 1 mM $MgSO_4$, no EDTA) which contained 30 g l^{-1} sucrose, 9 g l^{-1} Gelrite®, 1 mg l^{-1} 2.4-D and 4 mM $AlCl_3$ (0.8 mM Al^{3+}), at pH 3.6. In some experimental treatments the mitotic inhibitor colchicine (0.75 and 2.5 mM) was filter-sterilised and added to medium MM4 after autoclaving. Media (20 ml) were dispensed into 65 mm Petri dishes and callus was maintained in the dark for 2 weeks on Al^{3+}-free media, media containing Al^{3+} and media with colchicine.

4.2.3 Growth measurement and cell counts

The growth rate of callus was calculated after 2 weeks using the recorded fresh mass: (g g^{-1} $week^{-1}$, $fm_{week\ 2} - fm_{week\ 0} / fm_{week\ 0} / 2$). The dry mass of callus was determined after 2 weeks by incubating callus clumps in an oven (80 °C) for 1 h. The number of meristematic and non-embryogenic cells was determined before and after exposure to 0.8 mM Al^{3+} and the mitotic inhibitor colchicine (0.75 and 2.5 mM). Randomly selected calli (10 replicates / treatment) were placed into 5 ml of 0.4 M $K_2Cr_2O_7$, incubated at 80 °C for 30 min, shaken vigorously to break up any clumps and then placed on an orbital shaker (120 rpm) overnight. Thereafter the tubes were shaken by hand and 0.25 ml of the suspension was viewed using a Nikon Biophot light microscope (magnification 25x). The images were captured using Adobe Photoshop Version 5.0. The Kontron Image Analysis System (Ks

100, Release 3.0, Carl Zeiss, Germany, 1997) was used to count and measure the cells in the captured frame of view of ten replicates per treatment. Cell size (μm^2) was calculated by measuring the boundary of the cell wall using Ks 100. Three measurements per replicate were recorded for each randomly selected cell type, with a total of thirty measurements per cell type.

4.2.4 Determination of Al

Catechol violet method

Aluminium was determined using the catechol violet method (section 3.2.4) and by EDXM. Callus from both genotypes, that were exposed to media with or without Al^{3+}, was analysed. Glassware washed with 1M HCl was used throughout. Remnants of the gelatinous nutrient medium were removed using acid washed filter paper. The calli were dried for 1 hour at 80 °C, after which 0.1 g was incubated overnight in borosilicate glass tubes containing 3 ml HCl (10 M) and 1 ml HNO_3 (15 M), and digested in a Teflon block (120 °C for 30 min, 140 °C and 180 °C for 2 h each, and 190 °C for 3 h) until the volume was reduced to 1 ml. The digested samples were diluted to 5 ml and total Al in the callus mass was quantified by the catechol violet colorimetric method (section 3.2.4). The recovery of Al from a known standard showed that the efficiency of this system of analysis was 95% (range 86 – 105%, n = 12).

Energy Dispersive X-Ray Microanalysis

A portion of fresh callus (± 2 x 2 x 3 mm) was fixed onto a gold coated sample holder with Tissue-Tek® and rapidly frozen using liquid nitrogen slush (–210 °C) in an Oxford CT 1500 cryostation. The sample was transferred under vacuum to the cryochamber of the Philips Environmental Scanning Electron Microscope (XL 30 ESEM), at –182 °C. The frozen callus sample was fractured in the cryochamber, exposing both symplasmic and apoplasmic cellular detail to the electron beam. The fractured callus mass was transferred to the sample stage where it was freeze-dried (–70 °C) for 30 min. Thereafter the stage temperature was decreased to –187.1 °C and all readings were taken at this temperature. Samples were not coated with electrically conductive elements (i.e. Au or C) and a low accelerating voltage was used (15 kV). Energy dispersive X-ray microanalysis (EDXM)

spectra were recorded for the cell wall, cytomatrix and nucleus of callus cells when the total counts per second were greater than 300. Each spectrum was captured after 150 s and energy dispersive X-ray peaks were recorded from 1 to 9.7 keV, no elements were identified between 4 and 9.7 keV. Five spectra were recorded for each cell component per genotype and for each treatment. The elements detected (levels > 1000 ppm) were quantified using the EDX Standardless analysis approach (Goldstein *et al.*, 1992). For each analysis the elements detected were quantified on a weight % basis (Krüsemann, 2000). The calculated weight % for each element was normalised to 100% (i.e. the relative weight % for the 6 detected elements Al, P, S, Cl, K and Ca totaled 100%).

4.2.5 pH measurement

The pH of the callus and culture medium was recorded at week 0 (prior to exposure to Al^{3+}) and after 2 weeks, using a Mettler Toledo (INLAB 423) micro-electrode. The pH of the callus mass was recorded with the electrode tip immersed in its centre, and the pH of media by inserting the tip about 2 mm. The callus clump, situated in the middle of a 65 mm Petri dish, was removed once its pH was recorded, after which the medium pH was measured (i.e. directly below the callus clump, 10 mm and 30 mm away from the centre of the Petri dish).

4.2.6 Citric acid analysis

Approximately 0.3 g of fresh callus mass from both genotypes and treatments (control and 0.8 mM Al^{3+}) were analysed for citric acid. The fresh callus mass was ground in 4 ml (0.6 M) ice-cold perchloric acid ($HClO_4$) using a chilled glass pestle and mortar on ice, and then centrifuged at 5 °C and 5 000 g for 15 min. The supernatant was neutralised using 5 M KOH, incubated at 10 °C for 15 min and re-centrifuged for further 15 min, after which the supernatant was kept on ice. The citric acid content of the callus was determined spectrophotometrically at 340 nm (Beckman DU 500) by the amount of NADH oxidized in the conversion of citrate to oxaloacetate and pyruvate by citrate lyase (UV method, AEC Amersham PTY Limited, Moellering and Gruber, 1966). The citric acid content determined for the callus mass includes intracellular content as well as that excluded in the intercellular spaces and mucilaginous layer.

4.2.7 Statistical analyses

Data were tested for normality using the Kolmogorov-Smirnov test ($p > 0.05$). A One Way Analysis of Variance (ANOVA) was used to compare the results of two treatments and a Scheffé's multiple range test was applied when more than two variables were compared. Statistical significance between variables is denoted by dissimilar lower case alphabet characters or by three level of stars which corresponds with three p values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Also refer to section 2.2.3 in chapter 2.

4.3 Results

4.3.1 Al^{3+} and callus growth

The response of the Al-S and Al-R callus to Al^{3+} was evaluated in four separate experiments, using a selected treatment of 0.8 mM Al^{3+} and a 2 week culture time (Table 4.2). In all four experiments, in the absence of Al^{3+}, it appeared that the Al-R genotype maintained a significantly higher growth rate than the Al-S genotype (Expt. 1-3, $p < 0.0001$ and Expt. 4, $p < 0.05$). In each experiment Al^{3+} significantly decreased the callus growth rate of the Al-S genotype. The % growth inhibition was 42, 45, 65 and 50 % in the four experiments. In contrast, the growth of the Al-R genotype was not significantly affected by Al^{3+} and in one experiment (Expt. 4) Al^{3+} appeared to stimulate the growth of this genotype (Table 4.2). These results show that the response of the two *C. dactylon* genotypes to Al^{3+} is repeatable, with Al^{3+} inhibiting callus growth in the Al-S genotype and having no negative effect in the Al-R.

Fresh and dry mass measurements showed that in the Al^{3+} treatment, the Al-S callus had a significantly higher fresh/dry mass ratio than the control whereas Al^{3+} had no effect on this ratio in the Al-R genotype (Table 4.3). A possible explanation is that the Al^{3+}-treated callus of the Al-S genotype contained a greater number of non-embryogenic cells than the control, since Al^{3+} increases the rate of conversion of embryogenic meristematic callus cells into a non-embryogenic state (chapter 3, section 3.3.2). Non-embryogenic cells are large, elongated and highly vacuolated (chapter 2, section 2.3.2) and consequently contain higher water content than embryogenic cells.

Table 4.2 Comparison of callus growth rate from four separate experiments for two *C. dactylon* genotypes

Experiments	Al-S				Al-R			
	Control (C) $g\ g^{-1}\ week^{-1}$	Al^{3+} (A) $g\ g^{-1}\ week^{-1}$	Sig. Level (n)	A/C ratio	Control (C) $g\ g^{-1}\ week^{-1}$	Al^{3+} (A) $g\ g^{-1}\ week^{-1}$	Sig. Level (n)	A/C ratio
1	0.521 ± 0.038	0.304 ± 0.031	*** (20)	0.58 ± 0.06	0.784 ± 0.053	0.643 ± 0.049	NS (20)	0.82 ± 0.06
2	0.886 ± 0.092	0.487 ± 0.054	*** (22)	0.55 ± 0.06	1.51 ± 0.077	1.48 ± 0.05	NS (44)	0.98 ± 0.03
3	0.619 ± 0.036	0.219 ± 0.037	*** (14)	0.35 ± 0.06	1.046 ± 0.045	0.959 ± 0.057	NS (17)	0.92 ± 0.06
4	0.497 ± 0.025	0.2496 ± 0.019	*** (32)	0.50 ± 0.04	0.919 ± 0.029	1.044 ± 0.026	** (47)	1.14 ± 0.03

Callus was maintained for 2 weeks on nutrient media with (0.8 mM) or without Al^{3+} (control). The mean value (± SE) is presented. A One Way Analysis of Variance compared the means between the treatments for each genotype. The significance level was denoted as follows: ** $p < 0.01$; *** $p < 0.001$, NS = not significant, (n = sample size).

Table 4.3 The influence of Al^{3+} on callus fresh mass/dry mass ratio[1] in two *C. dactylon* genotypes

Growth measurement	Al-S			Al-R		
	Control	Al^{3+}	Sig. level (n)	Control	Al^{3+}	Sig. level (n)
Fresh mass (g)	0.0681 ± 0.002	0.0506 ± 0.001	*** (32)	0.0961 ± 0.002	0.1046 ± 0.002	** (47)
Dry mass (g)	0.0074 ± 0.0003	0.005 ± 0.0001	*** (29)	0.0118 ± 0.0002	0.0127 ± 0.0003	* (45)
Fresh/dry mass	9.25 ± 0.21	10.04 ± 0.18	** (29)	8.32 ± 0.08	8.31 ± 0.12	NS (45)

[1]Data from Experiment 4 (Table 4.2). The mean value (± SE) is presented. A One Way Analysis of Variance compared the means between the treatments for each genotype. The significance level was denoted as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, (n = sample size).

4.3.2 Al^{3+} and cell number and cell size

In order to further evaluate the cellular response with regard to cell number and cell size of the two genotypes to Al^{3+}, the same conditions (0.8 mM Al^{3+} and 2 week exposure period) were applied together with 0.75 or 2.5 mM colchicine. At week 0 (i.e. four-week-old callus), prior to Al^{3+} and colchicine treatments, the Al-R genotype contained about twice as many meristematic cells compared to the Al-S genotype (Table 4.4). Further, after the experimental period of 2 weeks on the control medium the meristematic cell number of the Al-S and Al-R genotypes doubled (1.66×10^6 - 3.53×10^6 and 3.32×10^6 - 7.9×10^6, respectively; Table 4.4). However, the number of Al-S meristematic cells was reduced by 88% with 0.8 mM Al^{3+} and by 71% and 95% with 0.75 mM and 2.5 mM colchicine, respectively. In the Al-R genotype only the higher concentration of colchicine (2.5 mM) resulted in any significant reduction (36%) in embryogenic cells. In both genotypes none of the treatments resulted in significant increases in non-embryogenic cell number, except for exposure to 2.5 mM colchicine in the Al-R genotype (Table 4.4).

At week 0 (i.e. four-week-old callus), although the sizes of the embryogenic cells of both genotypes were similar, the Al-S non-embryogenic cells were twice the size of the Al-R non-embryogenic cells (Table 4.5). In both genotypes only exposure to 2.5 mM colchicine for 2 weeks significantly increased cell size (both in meristematic and non-embryogenic cells) when compared with the control (Table 4.5). Also, in all treatments except 2.5 mM colchicine, the size of meristematic and non-meristematic cells was larger in the Al-S than the Al-R genotype.

Table 4.4 The influence of Al^{3+} and colchicine on meristematic and non-embryogenic cell number in two *C. dactylon* genotypes

Time (weeks)	Treatment	Al-S		Al-R	
		Meristematic cell number / ml (x 10^6)	Non-embryogenic cell number / ml (x 10^6)	Meristematic cell number / ml (x 10^6)	Non-embryogenic cell number / ml (x 10^6)
0	MS	1.66 ± 0.18	0.4 ± 0.04	3.32 ± 0.12	0.12 ± 0.04
2	SM	3.53 ± 0.47 b*	0.57 ± 0.36 a	7.9 ± 0.38 b	0.1 ± 0.05 a
	SM + 0.8 mM Al^{3+}	0.43 ± 0.1 a	1.24 ± 0.09 a	7.02 ± 0.35 b	0.43 ± 0.09 ab
	SM + 0.75 mM col**	1.03 ± 0.51 a	0.97 ± 0.21 a	6.73 ± 0.66 b	0.57 ± 0.27 ab
	SM + 2.5 mM col	0.16 ± 0.12 a	1.21 ± 0.11 a	5.04 ± 0.71 a	0.9 ± 0.16 b

Four-week-old calli cultured on unmodified nutrient medium (MS) were transferred onto screening media (MM4) and maintained in the dark for 2 weeks. The mean cell number (±SE) is presented. The means were compared within each genotype for each cell type using the Scheffé's multiple range test ($n = 10$, $p < 0.05$). b* Dissimilar alphabet characters within each column denote a statistical significance. Col** colchicine.

Table 4.5 The influence of Al^{3+} and colchicine on the size of meristematic and non-embryogenic cells in two *C. dactylon* genotypes

Time (weeks)	Treatment	Al-S		Al-R	
		Meristematic cell (μm^2)	Non-embryogenic cell (μm^2)	Meristematic cell (μm^2)	Non-embryogenic cell (μm^2)
0	MS	281 ± 19	1155 ± 167	222 ± 15	539 ± 34
2	SM	103 ± 6.7 a*	388 ± 42 a	64 ± 4.2 a	150 ± 9 a
	SM + 0.8 mM Al^{3+}	112 ± 7.7 a	740 ± 96 a	63 ± 4 a	258 ± 32 a
	SM + 0.75 mM col**	79 ± 4 a	684 ± 206 a	66 ± 2.5 a	157 ± 9 a
	SM + 2.5 mM col	306 ± 32 b	5036 ± 1307 b	247 ± 12 b	1284 ± 322 b

Four-week-old calli cultured on unmodified nutrient medium (MS) were transferred onto modified screening media (MM4) and maintained in the dark for 2 weeks. The mean cell size (±SE) is presented. The means were compared within each genotype for each cell type using the Scheffé's multiple range test ($n = 30$, $p < 0.05$). a* Dissimilar alphabet characters within each column denote a statistical significance. Col** colchicine.

4.3.3 Al^{3+} mobility

Uptake by callus mass

After 2 weeks exposure to 0.8 mM Al^{3+}, the fresh callus mass was dried, digested in acid and the total Al content of the dried callus mass was determined colorimetrically. Results show that a significantly higher concentration of Al was found in the Al-S than the Al-R genotype (Table 4.6). However these analyses could not determine whether Al^{3+} had entered the symplasm or whether it was confined to the apoplast. Further investigations were therefore conducted in order to ascertain whether Al^{3+} had entered the symplasm of the callus cells or whether it was confined to the intercellular spaces and the cell wall.

Table 4.6 Determination of total Al content of callus from two *C. dactylon* genotypes that were exposed to 0.8 mM Al^{3+} for 2 weeks

Measurement	Al-S	Al-R	Sig. level
Al content mM g^{-1} dry callus	10.92 ± 0.1	8.17 ± 0.3	***

The mean value (± SE) is presented. A One Way Analysis of Variance compared the means between the two genotypes. *** Significant difference between the means $p < 0.001$, $n = 12$.

Localisation of Al^{3+} in callus cells

Energy dispersive X-ray spectra were generated for Al-S and Al-R callus cells (Figure 4.1). Aluminium was detected in callus cells (cell walls, cytomatrix and nuclei) of both genotypes, only upon exposure to Al^{3+}. Quantification of X-ray spectra (Table 4.7) shows that: a) the cell walls of the Al-R genotype contained significantly more Al than those of the Al-S (20.6 and 13.7 %, respectively); b) Al content of the cytomatrix did not differ for cells of either genotypes; c) and nuclei of the Al-S cells contained about three times more Al than the Al-R cells.

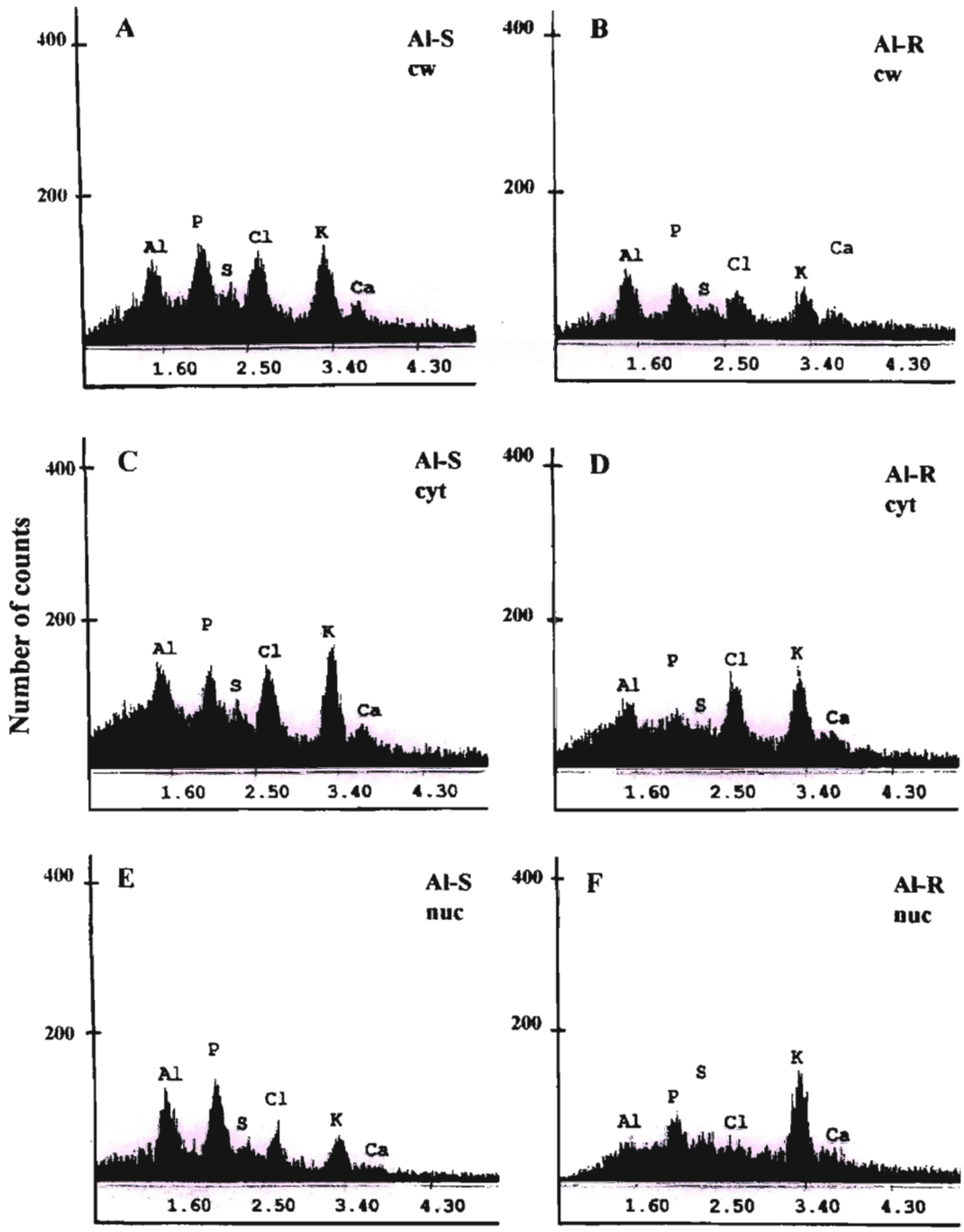

Figure 4.1 Representative EDXM spectra for Al-S and Al-R callus cells. Calli exposed to 0.8 mM Al^{3+} for 2 weeks were used to generate X-ray spectra for the cell wall (cw), cytomatrix (cyt) and nucleus (nuc).

Table 4.7 Determination of Al content in callus cells grown on media containing 0.8 mM Al^{3+} for 2 weeks, using X-ray microanalyses

Part of cell	Al content (wt %)		Significance level
	Al-S	Al-R	
cell wall	13.7 ± 1.3	20.6 ± 1	**
cytomatrix	11.7 ± 0.8	10.7 ± 2.2	NS
nucleus	21 ± 1.5	7.7 ± 0.9	***

The mean value (wt % ± SE) is presented. A One Way Analysis of Variance compared means between the two genotypes. The significance level was denoted as follows: ** $p < 0.01$; *** $p < 0.001$; NS $p > 0.05$, $n = 5$.

4.3.4 Al^{3+} exclusion

Immobilisation of Al^{3+} in the cell wall

Callus cells of the Al-R genotype contained significantly more Al (20.6%) in the cell wall than Al-S cells (13.7%). It is possible that properties of the cell wall of the Al-R genotype changed, thereby slowing down the transport of Al^{3+} ions across it and into the cytomatrix and nucleus. Present data support this hypothesis since more Al entered the Al-S cells (i.e. cytomatrix and nucleus) than those of the Al-R (Table 4.7). There was no difference in the cytomatrix Al^{3+} content, but there was significantly less Al^{3+} in the nucleus of the Al-R genotype when compared with the Al-S (Table 4.7).

pH barrier

The pH of the solution present in the intercellular spaces of the callus mass (including the mucilaginous layer) was measured, with care being taken to avoid the electrode from piercing the cell wall. Results are shown in Table 4.8 and indicate that at week 0 (i.e. after 4 weeks on callus induction medium and prior to Al^{3+} exposure) the Al-S callus exhibited a higher (pH 5.75) pH than the Al-R callus (pH 5.62). The pH of the callus induction medium ranges between 5.6 and 5.8, it therefore seems that the pH of the solution in the intercellular spaces of the callus and the culture medium is in equilibrium for each genotype, but with the Al-S possibly maintaining a higher pH than the Al-R. Callus of the Al-S and Al-R genotypes was transferred onto Al^{3+}-free screening medium at pH 3.6 for 2 weeks, this

resulted in decreased pH levels (when compared to week 0) for both genotypes, with the Al-R genotype continuing to show a significantly lower pH (Table 4.8). Following the 2 week Al^{3+} treatment the pH was lowered further in callus of both genotypes, however, the situation was reversed with the pH of the Al-R genotype being higher (pH 4.34) than that of the Al-S genotype (pH 4.08).

Table 4.8 pH measurements of callus cultured on media with and without Al^{3+}

Time (weeks)	Treatment	Measured pH of callus mass		Significance level (n)
		Al-S	Al-R	
0	callus induction	5.75 ± 0.02	5.62 ± 0.03	* (10; 10)
2	control	5.32 ± 0.08	4.9 ± 0.08	*** (22; 25)
	Al^{3+}	4.08 ± 0.03	4.34 ± 0.04	*** (22; 25)

Four-week-old callus was transferred onto screening media for 2 weeks that either excluded Al^{3+} or contained 0.8 mM Al^{3+}. The mean pH value (± SE) is presented. A One Way Analysis of Variance compared the means between the two genotypes. The significance level was denoted as follows: * $p < 0.05$; *** $p < 0.001$; (n: Al-S; Al-R).

At the beginning of the experiment the pH of all culture media was set at 3.6. After 2 weeks the pH of the Al^{3+}-free medium, supporting both the Al-S and Al-R genotypes, increased but showed no significant differences between the genotypes (Table 4.9). In the presence of Al^{3+}, the pH of medium supporting the Al-R genotype was higher than that of the Al-S medium, and this held true for all positions tested (distances relative to the callus at the centre of the Petri dish). Further, results indicate that there was a pH gradient in all media, with pH values decreasing with increasing distance away from the position of the callus mass.

Table 4.9 pH measurements of nutrient media with and without Al^{3+} (0.8 mM) after 2 weeks of culture

Treatment	Distance from callus (mm)	pH measured		Significance level
		Al-S	Al-R	
control	0	4.67 ± 0.08	4.52 ± 0.05	NS
	10	4.26 ±0.03	4.22 ± 0.02	NS
	30	4.12 ± 0.02	4.12 ± 0.02	NS
Al^{3+}	0	3.65 ± 0.04	3.90 ± 0.03	***
	10	3.52 ± 0.03	3.71 ± 0.02	***
	30	3.47 ±0.03	3.62 ± 0.02	***

The mean pH value (± SE) is presented. A One Way Analysis of Variance compared the means between the two genotypes. The significance level was denoted as follows: *** $p < 0.001$; NS $p > 0.05$; (n: Al-S = 22; Al-R = 25).

The concentration of Al^{3+} in the media was calculated for the range of pH values reported in Tables 4.8 and 4.9, using MINTEQA2. Figure 4.2 shows the relationship between increasing pH and Al^{3+} solubility, indicating a sharp decline in free Al^{3+} ions at pH values greater than 3.5. It appears, therefore, that according to MINTEQA2 predictions the 0.26 pH unit increase in the Al-R callus mass corresponds to a 5.4 times reduction in Al^{3+} concentration (Table 4.10). Further, the medium supporting the Al-R genotype had a 5.9 times lower Al^{3+} concentration in the centre of the Petri dish than the Al-S medium. These differences in Al^{3+} availability for the Al-S and Al-R genotypes were significant when calculated for both the callus mass and the nutrient media (Table 4.10).

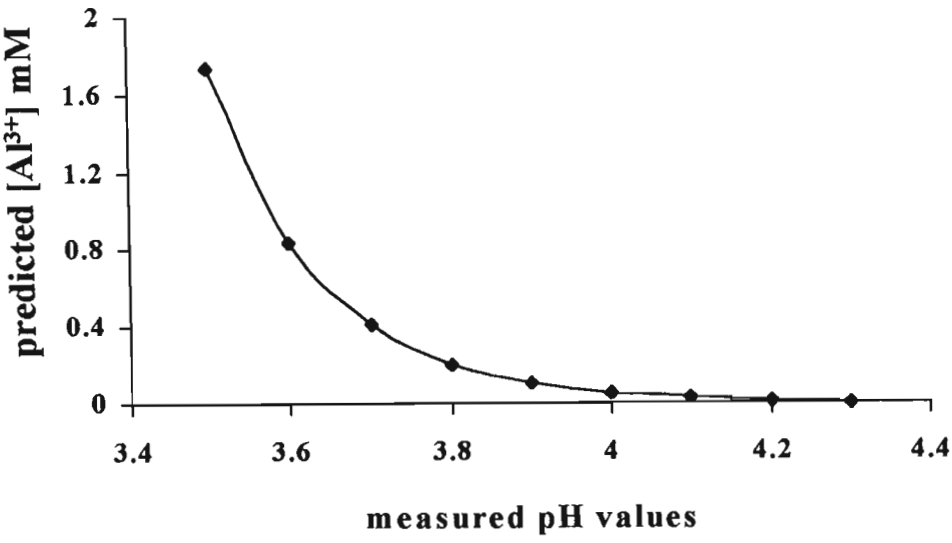

Figure 4.2 The relationship between measured pH and Al^{3+} availability in the nutrient media. The predicted Al^{3+} concentration was calculated using MINTEQA2.

Table 4.10 A comparison of measured pH and predicted Al^{3+} concentration for the callus mass and growth medium of two *C. dactylon* genotypes after 2 weeks of culture

Measurement	Al-S	Al-R	Significance level
callus pH	4.08 ± 0.03	4.34 ± 0.04	***
predicted [Al^{3+}] mM	0.0462 ± 0.01	0.0086 ± 0.002	***
medium pH (below callus)	3.65 ± 0.04	3.90 ± 0.03	***
predicted [Al^{3+}] mM	0.942 ± 0.15	0.161 ± 0.03	***

The predicted [Al^{3+}] was calculated, using MINTEQA2, for the mean pH values taken from Tables 4.8 and 4.9. The mean measured value (± SE) is presented. A One Way Analysis of Variance compared the means between the two genotypes. The significance level was denoted as follows: *** $p < 0.001$; (n: Al-S = 22; Al-R = 25).

Production of citric acid

It was hypothesized that if increased citric acid production was detected in the callus mass (i.e. callus cells, mucilaginous layer and solution of the intercellular spaces), then this organic acid would be able to chelate Al^{3+} and prevent it from inhibiting normal cell functioning. Enzymatic analyses of fresh callus revealed that neither genotype showed a significant change in citric acid production in response to Al^{3+} (Table 4.11).

Table 4.11 Citric acid content of callus from two *C. dactylon* genotypes cultured for 2 weeks on media with (0.8 mM) or without Al^{3+}

Treatment	Concentration of citric acid (μM g^{-1} fresh mass)		Significance
	Al-S	Al-R	level
Control	172.8 ± 37.3	201 ± 49.3	NS
Al^{3+}	144.7 ± 31.7	99 ± 14.7	NS
Sig. level	NS	NS	

The mean concentration (± SE) is presented. A One Way Analysis of Variance compared the means between treatments within each genotype and between the two genotypes. NS $p > 0.05$, $n = 10$.

4.4 Discussion

Differential root growth patterns in the presence of Al can be used to discriminate between Al-R and Al-S individuals (Matsumoto, 2000). For example when exposed to Al (30 μM for 24 h) root growth in maize was reduced by 70% and 40% in Al-S and Al-R cultivars, respectively (Pintro *et al.*, 1998). Maize root growth inhibition has been linked to decreased mitotic activity, where increasing Al concentration resulted in decreased number of dividing cells (Liu and Jiang, 2001). Similarly in this work it was also possible to differentiate between callus of two *C. dactylon* genotypes in relation to Al resistance upon a 2 week exposure to 0.8 mM Al^{3+} where the Al-S genotype exhibited a significantly lower growth rate of 42-65% compared to the control, whereas the Al-R callus remained unaffected (Table 4.2). This observed decreased growth rate of the Al-S callus appears to have been caused by a slowing down in mitotic activity, indicated by the 88% reduction in

meristematic cell number compared with the control (Table 4.4). It appears therefore that Al^{3+} inhibits mitosis, an effect that is evident both in the meristematic callus and root tip cells.

In the absence of Al^{3+}, the Al-R genotype had a significantly higher growth rate and twice as many meristematic cells when compared to the Al-S genotype (Table 4.4). It is not known whether this higher mitotic rate in the Al-R genotype makes this genotype in some way more resistant to Al^{3+} than the Al-S genotype. In a recent study it was suggested that in addition to mechanisms of Al exclusion, increased mitotic rate could serve as another possible mechanism of Al-resistance in wheat (Rayburn *et al.*, 2002). Those authors proposed that an overall increase in mitotic efficiency would reduce the negative effects of Al on cell division. Thus, it is possible that a similar Al-resistance strategy is evident in the Al-R genotype.

In the absence of Al^{3+}, the fresh mass of meristematic callus cells of both genotypes doubled in the first 2 weeks of culture (Table 4.4), indicating that callus was in an active growth phase. Yamamoto *et al.* (1994) established that log phase tobacco cells (i.e. undergoing rapid cell division) were most sensitive to Al and others have showed that Al uptake was critically dependent upon cell growth (Vitorello and Haug, 1996). In the present work, actively growing Al-S and Al-R callus cells take up Al (Figure 4.1, Table 4.7).

Some previous studies using EDXM have failed to locate Al in the nuclei of Al-stressed root apex cells (Marienfeld and Stelzer, 1993; Marienfeld *et al.*, 1995). Those authors reported that Al was located predominantly in the cell walls of peripheral cells in frozen-hydrated root tips of *Avena sativa*. They suggested that the plasma membrane was an effective barrier for Al in root apex cells. In contrast, Crawford *et al.* (1998) using EDXM on freeze-substituted roots of an Al-S (*Danthonia linkii*) and an Al-R (*Microlaena stipoides*) species showed that the majority of Al was accumulated in the nuclei. Also, cells from the Al-S species accumulated significantly more Al than those from the Al-R species. Further, the increased Al content in the nuclei of the Al-S cells was interfering with cell division and consequently inhibiting root growth (Crawford *et al.*, 1998). Similarly in this

work EDXM showed that Al was located in the nuclei of both the Al-S (Figure 4.1E) and Al-R (Figure 4.1F) callus cells. The Al-S genotype, which was the more negatively affected in terms of meristematic cell number (Table 4.4), was also found to contain considerably more Al than the Al-R genotype (Table 4.7). If Al inhibits DNA replication, (Clarkson, 1965; Matsumoto *et al.*, 1976) then this effect would be expected to be more pronounced in Al-S cells. Other researchers using lumogallion also reported links between Al accumulation in the nucleus of *Glycine max* root apex cells and inhibited cell division (Silva *et al.*, 2000). They reported that Al accumulated in the nuclei of meristematic cells of soybean root tips, but to a greater extent in Al-S than in Al-T genotypes.

Microtubules have been also identified as another potential intracellular target of Al^{3+} (Matsumoto, 2000; section 1.5). In this study a microtubule-disrupting agent (colchicine) was introduced to culture media in order to assess whether Al had a similar response to that of colchicine. Colchicine interferes with mitosis either by binding to tubulin and preventing microtubule assembly (Borkird and Sung, 1991) or through depolymerization of microtubules (Schibler and Huang, 1991). Exposure of the Al-S cells to a low level of colchicine resulted in reduced meristematic cell number with no significant effects on cell size with three-fold increase in colchicine concentration, however, the reduction in Al-S cell number was accompanied with an increase in cell size (Tables 4.4 and 4.5). The Al-R genotype was resistant to the low level of colchicine as both its cell number and size remained unaffected. However this resistance to colchicine did not persist with an increase in concentration, shown by reduced meristematic cell number and increased cell size (Tables 4.4 and 4.5).

The cytoskeleton (microtubules and actin filaments) plays a key role in determining cell shape and size (Smith, 2003) and facilitating cell division (Wasteneys, 2002). Therefore a disruption of the microtubules results in a change in cell shape and size. In this work a high level of colchicine inhibited mitosis as indicated by cell numbers (Table 4.4) and was associated with cell enlargement in both genotypes (Table 4.5), a result that is consistent with that of a microtubule disrupting agent (Borkird and Sung, 1991). The low level of colchicine interfered with division as indicated by cell number but did not increase cell size

in the Al-S genotype. This response of the Al-S cells to colchicine is similar to that resulting from exposure to Al^{3+}. Thus it is possible that a low level of colchicine and Al^{3+} affected cells in a similar manner (i.e. disrupting the microtubules and inhibiting cell division but not stimulating cell enlargement). Some researchers have shown that Al is located preferentially in actively growing cells: 1) cells of the DTZ in the maize root apex (Sivaguru *et al.*, 1999a); 2) log phase tobacco cells (Sivaguru *et al.*, 1999b; Schwarzerová *et al.*, 2002); and 3) meristematic wheat cells (Frantzios *et al.*, 2000). Those authors further reported that Al toxicity and inhibited cell division was attributed to depolymerized microtubules. In the present study, it is possible that inhibited cell division in the Al-S genotype occurred as a result of disruptions in the microtubules of the meristematic callus cells. However, further studies focused on the cytoskeleton will have to be conducted in order to confirm this possible Al-induced effect.

In this work the Al-R callus cells contained lower Al (Table 4.6) and the callus growth of this genotype was much less inhibited by Al^{3+} than that of the Al-S genotype (Table 4.3). It has been proposed that one way cells are able to exclude Al^{3+} is by reducing its solubility through the raising of the extra-cellular pH (Kochian, 1995; Matsumoto, 2000). However, in most published work pH changes have not been possible to determine as measurements were done in bulk nutrient solutions (Sivaguru and Paliwal, 1993; Pellet *et al.*, 1997). To date the most convincing evidence in support of increased root surface pH was demonstrated by mutant Al-R *Arabidopsis thaliana* roots which showed a 0.15 unit higher pH than the sensitive wildtype (Degenhardt *et al.*, 1998). Results obtained in the present study (Tables 4.8-4.10) indicate that under Al^{3+} stress the Al-R genotype maintained a pH in the intercellular spaces in the callus mass that was 0.26 units higher than that of the Al-S genotype (Table 4.8). In addition to increased callus pH, the Al-R genotype was also able to maintain a relatively higher pH of the culture medium (Table 4.9). As the pH of a medium increases, Al speciation rapidly changes from the toxic Al^{3+} species to the less toxic Al-hydroxides and Al precipitates, such that small pH differences can result in significant decreases in Al^{3+} toxicity (Kinraide, 1997; Figure 4.2). It is suggested, therefore, that such a strategy to overcome Al^{3+} toxicity is demonstrated by the callus cells of the Al-R *C. dactylon* genotype. Although there are a number of possible mechanisms by which root-

mediated changes in rhizosphere pH may occur (Hinsinger *et al.*, 2003), specific mechanisms which may account for these external pH differences in the Al-S and Al-R callus were not investigated further.

The quantification and localisation of Al in callus cells (Tables 4.6 and 4.7) provided evidence for a second possible mechanism of Al exclusion. In root apex cells Al can accumulate in the cell walls; since the trivalent Al^{3+} cation can bind to fixed negative charges (Silva *et al.*, 2000; Vázquez, 2002). In callus cells, EDXM spectral analyses showed more wall-accumulated Al in Al-R callus than for the Al-S cells (Table 4.7). Further this Al^{3+} immobilisation in the cell wall might have acted to slow down its transport into the cell since less Al entered the Al-R cells (cytomatrix and nucleus) than those of the Al-S (Table 4.7). This contrasts with findings for wheat (Delhaize *et al.*, 1993a) and soybean (Silva *et al.*, 2000) where no Al was found to accumulate in the walls of root tip cells. In other studies with maize, inhibition of root growth has been attributed to the reduced permeability of the cell wall to large molecules, such as proteins, by the formulation of a complex between Al and pectin (Schmohl and Horst, 2000). However, the accumulation of Al in the cell wall of Al-R callus cells had no apparent deleterious effect since there were no significant decreases in either meristematic cell number (Table 4.4) or callus growth rate (Table 4.2) when compared with the control. Further, even though there were no differences in the Al content of the cytomatrix between the two genotypes tested in this study (Table 4.7), the Al-S genotype accumulated three times more Al in the nucleus compared with the Al-R genotype. Thus it can be hypothesized that in addition to accumulating Al^{3+} in the cell wall, the Al-R genotype may also be able to compartmentalise Al intracellularly. Thus, although not tested in this study, it is also possible that symplasmic detoxification may have occurred in these callus cells.

The majority of work regarding Al resistance has centred around the exudation of Al chelator ligands or organic acids (Jones, 1998; Kochian *et al.*, 2002). Of those detected (malate, citrate, oxalate), citrate appears to be most commonly exuded by plant species in response to Al^{3+}. Further, citrate is regarded as being a more effective Al-chelating-ligand than malate (Hue *et al.*, 1986) and, apart from studies on wheat, malate alone has not been

implicated in the differential Al tolerance of any other member of the Poaceae (Jones, 1998). In this work the citrate level measured would include internal citrate as well as any citrate that was exuded from the cell and contained in the intercellular spaces and mucilaginous layer. Exposure to Al appeared to have no stimulatory effect on citric acid production as no change in citrate content of cells and mucilaginous layer was detected when compared to the control (Table 4.11). Although results in the present study appear to suggest that increased citric acid production is not a mechanism of resistance employed by the callus cells of the Al-R genotype, further investigation is warranted since the internal and external (exuded) citrate were not determined separately. Further, there are conflicting reports as to whether increased internal citrate levels of root cells imply increased efflux. Some researchers have shown this (Koyama *et al.*, 1999; Li *et al.*, 2002) while others have found that increased internal citrate levels is not associated with efflux rates (Watt and Evans, 1999; Delhaize *et al.*, 2003).

In the results presented in this chapter, Al^{3+} reduced callus growth in the Al-S genotype through an inhibition in cell division. This resulted possibly from an Al-induced interference in DNA synthesis or through disruptions in the functioning of the microtubules in the Al-S meristematic callus cells. However, the Al-R cells appeared to resist these negative effects of Al^{3+} and maintained a high callus growth rate. It appeared that these cells were able to maintain an efficient mitotic rate through avoidance of high Al in the nucleus compared to Al-S cells. This may have been achieved through an increase in external pH and chelation of Al in the cell wall. Taylor (1995) and Kochian (1995) suggested that since plants are complex multigenic systems it is unlikely that a single resistance strategy is responsible for Al avoidance. In support of this hypothesis some researchers have shown multiple resistance strategies in Al-R grasses and cereals (Pellet *et al.*, 1997; Wenzl *et al.*, 2001; Piñeros *et al.*, 2002; Vázquez, 2002). Although evidence for only two mechanisms of Al-resistance has been provided in the present study, it is possible that others (i.e. compartmentalisation of Al^{3+} in the cytomatrix) which were not investigated here are also evident in these meristematic callus cells. Results of the present study therefore suggest that undifferentiated meristematic callus cells potentially adopt a

multifaceted approach towards Al resistance, a strategy that appears to be evident in whole plants.

CHAPTER 5: FINAL DISCUSSION

5.1 Why an *in vitro* approach?

The majority of research into Al resistance has been carried out using the roots of whole plants. It has been shown that the root tip is the most Al-sensitive zone of the plant and that exposure to Al^{3+} results in root growth inhibition (Delhaize and Ryan, 1995; Kochian, 1995; Čiamporová, 2002). In this study an alternative approach was investigated, a group of actively dividing cells (callus) was exposed to Al. This was done in order to improve the understanding of the influence of Al on meristematic cells and with the possibility that information gained from the response of one cell type to Al^{3+} could be used to decipher the complex network of reactions and interactions involved during the whole plant's response to Al (Taylor, 1995; Matsumoto, 2000).

In the field of Al research *in vitro* plant cell culture techniques have been used in three main areas: (1) studies on the mechanisms of Al cytotoxicity and resistance, (2) induction of somaclonal variants (potentially Al-R) and (3) screening for resistance to Al^{3+} (Samac and Tesfaye, 2003). The present study contributes to the first and third areas of *in vitro* Al research. Callus was initiated from young *C. dactylon* leaves (Chapter 2). The *in vitro* approach allowed the monitoring of the physiological and biochemical reactions of one cell type (undifferentiated meristematic callus cells) to Al^{3+} and investigations into the effects of Al^{3+} on the growth rate (Chapter 3), number and size (Chapter 4) of callus cells. It was also possible to establish the movement of Al^{3+} and measure pH differences and citric acid production in these cells (Chapter 4).

Researchers have used callus and cell suspension cultures to investigate the responses of meristematic cells to Al (Chapter 3, Table 3.2). The physiological growth conditions of callus and cell suspension cultures are more easily adjusted and optimised than those of whole plants in nutrient solution (Matsumoto *et al.*, 2001). This work further established the advantages of the *in vitro* approach where the exposure of callus cells to Al^{3+} could be achieved on a nutrient medium that contained all the essential macro- and micro- nutrients required for callus growth (Chapter 3). Further, the *in vitro* growth environment is sterile,

thus excluding the potential interference of bacteria and fungi on the Al^{3+} response. The gelatinous nutrient medium supporting callus growth also facilitates the measurement of extra-cellular pH in various locations (with regard to the position of the callus cells), unlike a nutrient solution. In addition to studies on the mechanisms of Al^{3+} toxicity, the established somatic embryogenesis protocol can be used to regenerate potentially Al-R plants from the callus cells.

This work has shown that both nodal and leaf explants can be used to regenerate *C. dactylon* (chapter 2). These alternate means for the vegetative propagation of this grass is important since it allows for the multiplication of genotypes of interest (i.e. Al-resistant). *In vitro* propagation practices are, however, not seen to replace existing vegetative propagation programmes but rather to complement them. For example, the initial bulking-up of the genotype of interest could be done using *in vitro* regeneration protocols (chapter 2) and subsequent multiplication could be achieved using traditional vegetative methods.

In the absence of a viable inflorescence, as may occur in *C. dactylon* (section 1.7) standard sexual breeding practices are not possible. Therefore, in order to manipulate the genes of *C. dactylon* for the production of new varieties, *in vitro* techniques would have to be used. Those techniques include somaclonal variation and genetic engineering, and both approaches are reliant upon an *in vitro* regeneration system. For example, the prolonged culture of *Oryza sativa* callus on media containing Al resulted in somaclonal variation and the regeneration of Al-resistant rice genotypes (Van Sint Jan *et al.*, 1997). Genetic manipulation resulting in resistance to Al has also been achieved through the insertion of a foreign gene (bacterial citrate synthase) into tobacco (De la Fuente *et al.*, 1997) and carrot cell suspension cultures (Koyama *et al.*, 1999). Similar initiatives could be investigated in order to introduce Al^{3+}-resistance into genotypes of *C. dactylon*.

In addition, molecular biology initiatives could be used to assess gene expression in *C. dactylon* callus cells under Al stress. As discussed in the introduction (Chapter 1) the molecular basis of Al tolerance in plants is not completely understood and genes controlling Al^{3+}-resistance are still being investigated. It seems that genes controlling

general plant stress response are common to those expressed during Al stress (Milla *et al.*, 2002). During the process of callus initiation and growth these meristematic cells are exposed to a number of stresses (Fehér *et al.*, 2003). It is suggested, therefore, that exposure of callus cells to Al^{3+} could contribute towards the identification of Al^{3+}-resistance genes.

Aluminium-resistant *C. dactylon* genotypes resulting from conventional or *in vitro* screening methods need to be conserved for both current and future use. Existing *ex situ* conservation plans include seed storage and field genebanks. Alternative strategies need to be developed since most hybrid and transgenic genotypes do not produce seeds. Also, plants grown in fields are at risk from pests, diseases and other natural hazards. *In vitro* techniques offer alternative strategies for the conservation of germplasm in a controlled, pest- and disease-free environment. For example, some of the initial research regarding the development of *in vitro* conservation protocols has been done on forestry and fruit tree species (Withers and Engelmann, 1998; Lambardi and De Carlo, 2003).

In vitro conservation techniques include slow growth for short- to medium-term storage and cryopreservation (in liquid nitrogen at -196 °C where cellular activities are arrested) for long-term storage. Shoot tips and nodal cuttings are explants most frequently employed for short-term storage (Withers and Engelmann, 1998; Lambardi and De Carlo, 2003). The organogenesis protocol established in the present study provides a potential *in vitro* system for the slow growth storage of Al-resistant *C. dactylon* genotypes. In addition, the potential of conserving *C. dactylon* germplasm in the long-term through the cryopreservation of somatic embryos (resulting from the somatic embryogenesis protocol developed in this work) could also be investigated. The *in vitro* propagation protocols established in this study thus provide an important framework for the potential conservation of desired *C. dactylon* genotypes.

5.2 Can the response of meristematic callus cells be used to define that of the parent plant?

Some researchers have suggested that in order to understand the complex network of interactions and reactions that define the whole plants response to Al, investigators need to use whole plants (Kochian, 1995; Bennet and Breen, 1991; Čiamporová, 2002). Although the use of callus and cell suspension cultures provide useful information on the effects of Al on meristematic cells (Matsumoto, 2000), this approach is only able to assess the influence of Al on one cell type. It has been shown that there is diversity in the response of root cells to Al toxicity with respect to their tissue type, developmental stage and position within the root (Čiamporová, 2002). Callus and cell suspension cultures are unable to investigate the effects of Al on the different cell types present in root tissue.

In addition, it appears that the position of cells in the root tissue influences response to Al (Čiamporová, 2002). For example, in a study using maize roots it was reported that Al had no influence on the orientation of microtubules in the cells of the epidermis and outer cortex but was observed to depolymerise the microtubules of cells in the inner cortex (Blancaflor *et al.*, 1998). In another study on maize, researchers found that microtubules of the peripheral root cortex were depolymerised before those of the epidermis (Sivaguru *et al.*, 1999a). It has been suggested that the early response of the inner root cells (located at a greater distance from the Al source) must be mediated by some signal transduction pathway which influences alterations in the cell without direct contact with the stress (Čiamporová, 2002). It is suggested, therefore, that an unorganised mass of dividing cells (callus) will be unable to investigate the positional effects of cells with regard to Al toxicity.

The diversity in response to Al of cells of different tissue and developmental stages can be expected since the specific physiological and biochemical characteristics of the cells differ (Čiamporová, 2002). However, in some studies it has been shown that individual cells of the same tissue respond differently to Al. For example in the maize root cortex, single cells or a small number of cells within one cell file were severely damaged while the neighbouring cells were unaffected (Čiamporová, 2000). In another study on maize, Vazquez (2002) reported a similar finding with those cells of the root cortex having

accumulated Al. Lysigenous cavities were formed in the root cortex by the death of those Al hyperaccumulator cells, thereby excluding Al from the neighbouring cells. It seems that under Al stress certain cells are programmed in some way to accumulate Al and then undergo rapid disintegration, thus enabling the rest of the tissue to survive (Čiamporová, 2000; Vazquez, 2002). It seems unlikely that this mechanism of Al exclusion could be investigated using callus cells.

Roots have been identified as being more sensitive to Al than shoots and therefore much research has been focused on this organ of the whole plant (Delhaize and Ryan, 1995; Rout *et al.*, 2001; Barcelo and Poschenrieder, 2002). However, some researchers have suggested that for an improved understanding of the whole plants' response to Al, investigations need to move beyond the root apex (Kochian, 1995; Bennet and Breen, 1991; Čiamporová, 2002). Towards this end workers in the Al field have focused their efforts on the whole plants response to Al, in particular the shoots. Some researchers have proposed that shoots may play an important role in Al resistance (Yang *et al.*, 2001; Li *et al.*, 2002). They found that Al-induced citrate secretion decreases when shoots are excised from intact plants (Yang *et al.*, 2001; Li *et al.*, 2002). Those authors suggested that shoots may not be supplying citrate to the root apex cells but rather a source of carbon (energy) for citrate synthesis. It is also possible that certain components involved in citrate synthesis may be produced in the shoots and transported to the root apex cells. Meristematic callus cells will not be able to investigate such a response to Al. The *in vitro* system developed in the present study allows for the interaction of Al with one cell type and does not facilitate investigations into relationships, concerning Al, amongst different cell types, tissues and organs present in the whole plant.

5.3 Can undifferentiated callus cells be used to investigate mechanisms of Al^{3+} toxicity and resistance?

In the somatic embryogenesis protocol established in the present study, the MS nutrient medium supplemented with an auxin (2,4-D) was used to initiate callus from the basal segment of young *C. dactylon* leaves (Chapter 2). The auxin 2,4-D and the *in vitro* stress of

wounding are factors that influence dedifferentiation. Dedifferentiation is characterised by remarkable changes in the pattern of gene expression as cells switch from a programme that drives the functions of a leaf cell to a new one directing re-entry into the cell cycle, thereby stimulating cell division and the production of callus (von Arnold *et al.*, 2002; Fehér *et al.*, 2003; Grafi, 2004). It has been suggested that during this process existing transcriptional and translational profiles are erased in order to allow cells to set a new developmental programme (Fehér *et al.*, 2003). It is possible that during genetic, metabolic and physiological reprogramming of these leaf cells that gene/s governing the plants' stress response (resistance to Al) could be switched-off or their expression altered. Furthermore, it is also possible that Al^{3+}-resistance genes are tissue-specific and only switched-on in cells of the Al-sensitive root tip.

Consequently, one of the main concerns of using undifferentiated cells for investigating Al toxicity and resistance is, therefore, that genes governing Al resistance may not be expressed in these cells. Some researchers (Grosset *et al.*, 1990; Pasternak *et al.*, 2002) have argued against this potential loss of stress resistance and stated that during dedifferentiation most genes related to stress response are in fact operational and play a key role in initiating and maintaining cell division. Furthermore, as discussed in section 1.8, genes expressed during Al stress are common to those present during general plant stress (Milla *et al.*, 2002). For example, recent studies using maize (Boscolo *et al.*, 2003) and pea plants (Yamamoto *et al.*, 2003) have shown links between Al and oxidative stress. Indeed, it has been suggested that some of the genes expressed during Al stress are also evident during oxidative stress (Richards *et al.*, 1998; Milla *et al.*, 2002; Watt, 2003).

In vitro tissue culture conditions expose leaf explants to a significant stress as they are removed from their original tissue environment and placed on nutrient media containing non-physiological concentrations of growth regulators and macro- and micro- nutrients (Fehér *et al.*, 2003). It is, therefore, suggested that genes governing the plant's stress response (including Al^{3+}-resistance) are expressed in undifferentiated callus cells. Results of the present study appear to support that suggestion since the response of callus from the genotypes to Al^{3+} was different (Figure 3.7). Aluminium inhibited the growth rate of the

Al-S callus but had no negative effect on the Al-R callus. It seemed, under the experimental conditions used, that the Al-R callus cells were able to avoid the negative effects of this metal. This was achieved by an increase in extracellular pH (Tables 4.8 and 4.9) and a chelation of Al^{3+} in the cell wall (Figure 4.1 and Table 4.7). These strategies of Al^{3+}-avoidance have been previously demonstrated for roots of whole plants. It appears, therefore, that genes controlling Al^{3+}-resistance are probably unaltered and expressed in these undifferentiated callus cells. The present study confirms that resistance to Al is mediated at the cellular level and that callus and cell suspension cultures provide a useful tool for the investigation of mechanisms of Al^{3+} resistance, as have been reported in many other studies (Ojima and Ohira, 1983; Conner and Meredith, 1985b; Parrot and Bouton, 1990; Devi *et al.*, 2001; Toan and Debergh, 2002).

Another concern of using callus cells is that mutations (i.e. somaclonal variation) may occur during cell division, resulting in the loss of Al^{3+}-resistance. Other workers have generally used the concept of somaclonal variation to generate novel phenotypes that are resistant to Al^{3+}. This was demonstrated using rice (Van Sint Jan *et al.*, 1997) and maize (Moon *et al.*, 1997) callus cultures. Somaclonal variation is generally defined as the phenotypic variation found in plants that have been regenerated *in vitro* via adventitious means (Joyce *et al.*, 2003). The resulting plants differ from the original parent plant if during growth and development the genetic material is rearranged. This variation is influenced by type of explant, developmental state of explant, plant growth regulators (mainly auxins) and the regeneration protocol (George, 1996; Cassells and Curry, 2001).

Other factors that influence somaclonal variation include ploidy level and genotype. According to Clark and Wall (1996) polyploid individuals are more susceptible to somaclonal variation than haploid and diploid cultivars. *Cynodon dactylon* is tetraploid, suggesting therefore that there is an increased chance of variation occurring during cell division. It is possible that during mitosis chromosomes may have crossed-over, DNA fragments may have changed position in the plant genome and genes governing Al^{3+}-resistance may have been silenced. If this occurs then the response of these callus cells to Al^{3+} will be altered and therefore not represent those of the parent plant. In this study,

somaclonal variation was minimised by the use of young leaf explants, a low concentration of 2,4-D (1 mg l^{-1}) and a short culture period (2 weeks) (Chapters 2 and 3). In this work somaclonal variation did not appear to alter the genes controlling Al^{3+}-resistance since the response of the Al-R callus to Al^{3+} was consistent, as shown in four separate experiments (Table 4.2).

In this work, the Al-S callus cells contained more Al than the Al-R cells (Table 4.6) and the nuclei of the Al-S cells contained three times more Al than the Al-R (Table 4.7). Furthermore, when exposed to Al^{3+} the Al-S meristematic cell number was reduced by 88% (Table 4.4). It seems therefore that Al^{3+} stopped cell division in the callus cells. The Al-S callus cells appeared to accumulate Al^{3+} in the nuclei (Table 4.7), suggesting therefore that inhibited mitosis may have resulted from a disruption in DNA synthesis. The present work supports the earlier findings of other researchers who adopted the *in vitro* approach to investigate Al toxicity. The inhibitory effect of Al on cell division has been demonstrated using alfalfa (Parrot and Bouton, 1990) and orange (Toan and Debergh, 2002) callus and tobacco (Yamamoto *et al.*, 1994), rice (Rahman *et al.*, 1999), red spruce (Minocha *et al.*, 2001) and barley (Pan *et al.*, 2002) cell suspension cultures. In whole plants, Al-inhibited root growth has been linked to the cessation of mitosis in the meristematic zone of the root tip (Matsumoto *et al.*, 2001; Čiamporová, 2002) with Al accumulation in the nuclei (Silva *et al.*, 2000; Liu and Jiang, 2001). It seems, therefore, that Al exhibits a similar inhibitory response (i.e. stops mitosis) in the meristematic cells of callus and the root tip.

Although Al^{3+} has been linked to the cessation of cell division, the specific mechanisms of Al^{3+} toxicity have not been elucidated. Towards this end many researchers have used cell suspension cultures to investigate the effects of Al (Ikegawa *et al.*, 1998; Sivaguru *et al.*, 1999b; Schmohl and Horst, 2000; Yamamoto *et al.*, 2002; Schwarzerova *et al.*, 2002). Some researchers using tobacco cell suspension cultures have been able to link inhibited cell division with an Al-induced disruption of the cortical microtubules (Sivaguru *et al.*, 1999b; Schwarzerova *et al.*, 2002). In this study, the response of callus cells to Al^{3+} and a low level of colchicine (mitotic inhibitor) are similar. It is suggested therefore that an Al^{3+}-induced inhibition in callus cell division may have resulted from a disruption (i.e.

depolymerisation) of the microtubules (Tables 4.4 and 4.5). However, this response needs to be investigated further to confirm this possible Al-induced effect.

The meristematic zone of the root tip represents an important growing point of the whole plant. Do whole plants offer extra or special means of defence for the root apex, since an efficient root system is critical for plant growth? In their reviews, Taylor (1995) and Kochian (1995) have proposed that whole plants are complex multigenic systems that would most likely employ several Al resistance strategies in order to protect the root meristem from Al injury. However, most researchers appeared to have focused on a single strategy, the release of an Al-chelating agent (mainly citrate) (Jones, 1998; Ma, 2000; Kochian *et al.*, 2002). Investigations on the release of organic acids in response to an Al stress in whole plants have provided evidence that suggests that meristematic cells of the root apex act independently with regard to perception of and response to the Al stress. Recent research has shown that Al-induced the release of citrate occurs only in the root apex cells (Yang *et al.*, 2001; Li *et al.*, 2002). Furthermore, the anion channels identified for citrate release were only detected in the meristematic cells and were absent in cells from the elongation zone and mature regions of the root (Kollmeier *et al.*, 2001). Those results suggest, therefore, that it is unlikely that neighbouring cells of the root contribute towards the protection of the meristem from Al injury. Results of the present work appear to offer some support to the hypothesis that meristematic cells are able to act independently with regard to perception of the Al toxicity and triggering a suitable Al-resistance strategy. In the presence of Al^{3+}, the growth rate of the Al-S callus was significantly reduced whereas that of the Al-R genotype was unaffected (Table 4.2). It appeared that in response to the Al stress, the Al-R callus cells are able to avoid Al injury by employing possible strategies that excluded Al from these cells (Tables 4.7-4.9, Figure 4.1) although no evidence is offered to support the role of organic acids.

Some researchers argued that the high level of Al-resistance evident in some species can not be explained only by the release of an organic acid. They concluded that it is more likely that those species adopt several Al exclusion strategies (Parker and Pedler, 1998; Wenzl *et al.*, 2001). In support of that hypothesis multiple Al avoidance mechanisms have

been demonstrated using whole plants of wheat (Pellet *et al.*, 1997), signalgrass (Wenzl *et al.*, 2001) and maize (Vázquez, 2002). Results of the present study provide evidence for two Al resistance strategies in that *Cynodon dactylon* callus cells appeared to be able to protect against Al-injury through an increase in extracellular pH and Al chelation in the walls of callus cells. To date these strategies for Al avoidance have only been demonstrated in whole plants. The present work has provided an experimental system to study the mechanisms of Al avoidance in meristematic callus cells and to further the comparison of Al^{3+} resistance in cells, organs and whole plants.

LIST OF PUBLICATIONS

The present study has led to the following publications:

1. Ramgareeb S, Cooke J.A and Watt M.P 2004 Responses of meristematic callus cells of two *Cynodon dactylon* genotypes to aluminium. Journal of Plant Physiology 161: 1245-1258.

2. Ramgareeb S, Watt M.P and Cooke J.A 2001 Micropropagation of *Cynodon dactylon* from leaves and nodal segments. South African Journal of Botany 67: 250-257.

3. Ramgareeb S, Watt M.P, Marsh C.A and Cooke J.A 1999 Assessment of Al^{3+} availability in callus culture media for screening tolerant genotypes of *Cynodon dactylon*. Plant Cell, Tissue and Organ Culture 56: 65-68.

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