



**An investigation into the role of sodium chloride
in the desiccation tolerance of
isolated *in vitro* axillary buds of *Eucalyptus grandis*.**

By

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PREFACE:

The experimental work carried out in this dissertation was conducted under the supervision of Prof. D. J. Mycock in the School of Animal, Plant and Environmental Sciences, at the University of the Witwatersrand (Johannesburg), and Prof. M. P. Watt in the School of Biological & Conservation Sciences, at the University of KwaZulu Natal (Durban).

I declare that the work submitted in this dissertation is the result of my own investigations, and the work of others has been acknowledged in the text.



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ABSTRACT

The ability of plant cells, tissues and organs to withstand desiccation is an important criterion for their successful cryopreservation. The aim of the present experiments was to test the hypothesis that exposure of *Eucalyptus grandis* *in vitro* shoot clusters to NaCl was associated with a cross triggering of desiccation tolerance. Shoots clusters were cultured on standard semi- solid nutrient Murashige and Skoog (1962)(MS) medium (40mM NO₃⁻ + 20mM NH₄⁺) containing 0 - 100mM NaCl, or modified semi- solid medium (40mM NO₃⁻ and no NH₄⁺) containing 0 - 100mM NaCl. The shoot clusters were also cultured in the presence of abscisic acid (ABA [5mg/l]), either as a pretreatment (5 days) or in combination with the standard/modified MS formulation (14 days). Axillary buds were isolated from the shoot clusters and dried over activated silica gel for 20, 40 and 60 minutes.

As the NaCl concentration increased the *E. grandis* shoots showed increasing signs of stress i.e. physical changes to the plants appearance. This was especially true for shoots cultured on the modified MS formulation. In all experiments, the ability of the isolated buds to lose water was reduced, possibly due to the compartmentalization of Na⁺ and Cl⁻ ions. Concomitantly the viability of the buds that were cultured on unmodified nutrient medium after being subjected to the standard/ modified nutrient medium with the addition of NaCl was greatly reduced after 20 minutes of drying (approximately 70% of the buds were dead).

The addition of ABA as a pretreatment resulted in an increase in the number of shoot clusters that experienced no injury (qualitative data) when cultured on the standard MS formulation. However, injury increased in the presence of the modified MS medium (40mM NO₃⁻). The water content for the plant tissue cultured on both the standard and modified MS medium indicated that there was no reduction in water loss, i.e the buds resisted water loss following desiccation (water content ranged between 59% after no drying to 49% after 20 minutes of drying) and again, the viability was compromised (approximately 70% reduction in survival). Prolonged exposure to ABA had damaging effects and this was exacerbated by the addition of NaCl. The water content of the isolated buds that were treated with ABA showed an insignificant change in water loss, but this was not sufficient to allow for cryopreservation. Hence, the hypothesis that NaCl acts as

a cross trigger for desiccation tolerance was rejected. Indeed *E. grandis in vitro* axillary buds are desiccation sensitive and exhibit avoidance characteristics in their response to water loss.

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ABBREVIATIONS AND SYMBOLS

°C	Degree Celsius
%	Percent
PGR	Plant Growth Regulators
ABA	Absciscic Acid
LEA	Late Embryogenesis Abundant
ROS	Reactive Oxygen Species
NaCl	Sodium Chloride
BAP	Benzlaminopurine
NAA	Naphthaleneacetic acid
IBA	Indole-3-butyric acid
h	hours
n	number
Ca ²⁺	Calcium ions
K ⁺	Potassium ions
Na ⁺	Sodium ions
Cl ⁻	Chloride ions
NO ₃ ⁻	Nitrate ions
NH ₄ ⁺	Ammonium ions
mM	milli molar
®	Registered
g.l ⁻¹	grams per liter
ml.l ⁻¹	milliliters per liter
mg.l ⁻¹	milligrams per liter
μ. E ⁻² s ⁻¹	micro Einsteins per meter square per second
MS	Murashige and Skoog nutrient medium (1962)
SAS	Statistical software
ANOVA	Analysis of Variance
pH	hydrogen ion concentration

1. INTRODUCTION

1.1 Background

The genus *Eucalyptus* forms a major component of the Australian flora, and belongs to the *Myrtaceae* family. The trees of this genus are generally characterised by long straight trunks that may reach heights of up to 90 meters, although members may also occur as multiple shrubby mallees (McComb and Bennett, 1986). The *Eucalypts* are classified by the operculum that covers the unopened flowers; this is where the genus derived its name (*eu*= well; *calyptos*= covered). Some members of the *Eucalypt* genus are economically important to the forestry industry and are used for the production of timber, fuel woods, and paper and pulp (Lakshmi Sita, 1986; Mabvurira and Pukkala, 2002; Raymond, 2002). The leaves also contain oils, which are utilized in the medicinal, industrial, and cosmetic sectors (McComb and Bennett, 1986). In certain regions *Eucalyptus* trees are used as wind and fire breakers, and have increasingly been used as ornamental plants in certain countries (McComb and Bennett, 1986).

Australia has the largest distribution of *Eucalyptus* plantations and members of this genus have been introduced into various other countries as exotic plants, e.g. Spain, USA, Iran, Brazil, Uruguay, Madagascar, Morocco, Portugal and South Africa (McComb and Bennett, 1986; Louw, 2004). In South Africa, approximately 37.7% of forestry land is covered by *Eucalyptus* plantations (Anon, 2008).

Eucalyptus grandis

Eucalyptus grandis is native to the east coast of Australia and is commonly known as the Rose Gum or Flooded Gum (Durand- Cresswell, *et al.*, 1982). *E. grandis* trees thrive best in warm, humid conditions, and are capable of surviving moderate to heavy frost and cold winds (Meskimen and Francis, 2009). In Africa, *E. grandis* is confined to the high rainfall areas in the mountains and along the southern and eastern coast of Southern Africa. The species is grown commercially on a larger scale than any other *Eucalypt* in Southern Africa (Meskimen and Francis, 2009). The sap from *E. grandis* is pale pink, with the heartwood being light to dark red (Meskimen and Francis, Accessed on 26th

January 2009) hence, its common name, Rose Gum. This species of *Eucalyptus* grows to an approximate height of 55 meters and has a canopy diameter of approximately 1.83 meters. The wood produced by *E. grandis* is of superior-quality, making it desirable for a wide variety of uses e.g. timber, pulp, and fuel woods. *E. grandis* is particularly suited for the production of high quality paper, due to its fast growing rate and superior fiber properties (Arbothnot, 2001). Due to the positive qualities of this species, there has been an increase in demand for more products being produced from the wood of *E. grandis*. Therefore the species is of great importance in the commercial forestry industry. Due to the great and increasing demand for wood from this genus and other *Eucalyptus* species, it is expected that there will be shortages of timber required for the paper mills in the near future. Mondi Group, South Africa therefore aims to increase the production of *E. grandis* so that consumer demands can be met.

1.2 Methods of Propagation

1.2.1 Traditional Methods

Various methods have been adopted in the propagation of *Eucalyptus* trees. Seeds are grown in seedling trays, where germination takes place 7-14 days after sowing (Joshi, *et al.*, 2003). Each seed has a different genotype (Das and Mitra, 1990) so there will be genetic variation between plants. Clonal propagation is therefore desirable. Grafting is costly and not suitable for plantation species (only suitable for ornamental plants) (Mehre- Palta, 1982; McComb and Bennett, 1986). The only suitable and successful conventional vegetative method is cuttings. Cuttings are generally the easiest and most commonly used method of propagating *Eucalyptus* trees. The cuttings are obtained from a juvenile tree or from seedlings as they generally root more easily (McComb and Bennett, 1986; Hammet, 1992; Meskimen and Francis, 2009). The selected genotypes are then used as clonal hedges from which new cuttings are generated. The disadvantages are that one parent plant generates only a few cuttings, and one cutting generates one new plant, and the rooting of these cuttings can be difficult with many clones particularly hybrids.

1.2.2 Tissue Culture and Micropropagation

Plant tissue culture can be described as the *in vitro* regeneration of whole plants from tissues, organs, and cells on/in a nutrient medium containing growth regulators, under aseptic conditions (Hammat, 1992; George, 1996; Loyola-Vargas, *et al.*, 2008). There are two main forms of development that may be used to regenerate plantlets, direct and indirect morphogenesis.

Direct morphogenesis involves the direct formation of shoots/roots or embryos in an organized manner from an isolated part of the parent plant. For example shoots with axillary nodes may be excised from the parent plant and placed on a nutrient medium (George, 1996). Depending on the composition of the medium shoot development can be stimulated from the axillary buds, and the shoots can be stimulated to produce roots, thereby producing a clonal plant with preserved genetic traits. Indirect morphogenesis, on the other hand involves the formation of callus (a group of undifferentiated cells). Callus cells can be induced to differentiate into whole plants via the formation of shoot/root systems (organogenesis) or via somatic embryo formation (embryogenesis) (Mycock, *et al.*, 2004).

There are various advantages to micropropagation over traditional vegetative propagation methods. The main advantage of micropropagation over cuttings is the production of many plants (George, 1993). Secondly, micropropagation of *Eucalypts* via *in vitro* techniques allows for better genetic uniformity (that is the production of plants that are identical to the parent plant), thus, conserving the desirable wood qualities (Withers, 1989).

Somaclonal variation is variation seen in plants that have been produced by plant tissue culture and is common in plants regenerated from callus. Somaclonal variation is undesirable, especially when regenerating genetically stable plants for long term storage (Kantha, 1985; Paulet, *et al.*, 1993). Axillary bud proliferation is the most common of the routes of regeneration because it is the simplest and quickest method. It has a further

advantage in that it is a true-to-type form of proliferation and therefore, there is no risk of somaclonal variation (i.e. it is a direct regeneration of plant tissues) (Kartha, 1985; Paulet, *et al.*, 1993).

1.2.3 Germplasm Conservation

In order to achieve and maintain the desired wood properties exhibited by selected forestry species including *E. grandis*, it is important to minimize the loss of genetic material. This can be achieved by plant germplasm conservation. There are two approaches by which the plant genetic material may be preserved (i) *in situ* and (ii) *ex situ* conservation.

In situ conservation refers to the preservation of species in their natural habitats (Krogstrup, *et al.*, 1992). The true *in situ* conservation of *Eucalypts* is not feasible in South Africa as the genus is exotic thus this method is restricted to Australia only. At present, the forestry industry does not maintain as wide a variation as possible for breeding programs as this is extremely costly in terms of land, water and maintenance. One way of overcoming this problem is through *ex situ* conservation.

Ex situ conservation is a common conservation method used for agricultural and commercial forestry species, which usually utilizes field plantations, seed stores, and gene banks (Watt, *et al.*, 2000; Benson, 2008). Commercial forestry utilizes trial planting, hedges, greenhouses and hydroponic systems (Figure 1.2 a-d) for the maintenance of active clonal collections of forest species (Watt, *et al.*, 2000a). Although this process of conserving plant germplasm is successful, the maintenance of the clonal collections is expensive and labour intensive (Watt, *et al.*, 2000a). The *in vitro* propagation and storage (Figure 1.2 e) of commercial forest species is an alternative to *ex situ* field banks. *In vitro* storage technology has the distinct advantage in that a variety of cells, tissues and organs may be stored in a very small area.

The *in vitro* conservation of germplasm is beneficial to the commercial forestry industry as a large number of selected *E. grandis* parent plants can be regenerated or cloned in a

relatively short period. Further, as the germplasm is maintained under controlled conditions, unlike field banks which are exposed to environmental hazards, there is less risk of loss (Withers, 1986; Baek, *et al.*, 2003; Panis and Lambardi, 2005). Other concerns that can be reduced/obviated with *in vitro* germplasm conservation include: the lack of suitable land, the minimal chance of being exposed to pests and diseases, and hazards such as weather damage, and vandalism (Baek, *et al.*, 2003; Panis and Lambardi, 2005).

In vitro storage can be classified into three categories: short-, medium-, and long term storage. Short- to- medium term storage (minimal growth procedures) involves the storage of actively growing cultures and can be useful when commercial micropropagation needs to be regulated according to customer demands. Plant stocks are maintained under slow growth conditions and can be returned to the active growth phase when necessary (Mycock, *et al.*, 2004). However, this process requires that plant material be regularly subcultured on fresh medium containing plant growth regulators and vitamins (Watt, *et al.*, 2000b). Using this approach, there is always a risk of contamination, human error and the risk of somaclonal variation, i.e. mutations that occur spontaneously in tissue culture and can increase with repeated subculturing (Panis and Lambardi, 2005).

In vitro storage of *E. grandis* has been achieved for short- and medium term periods (Watt, *et al.*, 2000b). Long-term storage on the other hand involves a process called cryopreservation, and has however, not yet been achieved for *Eucalyptus* types important to the South African forestry industry.

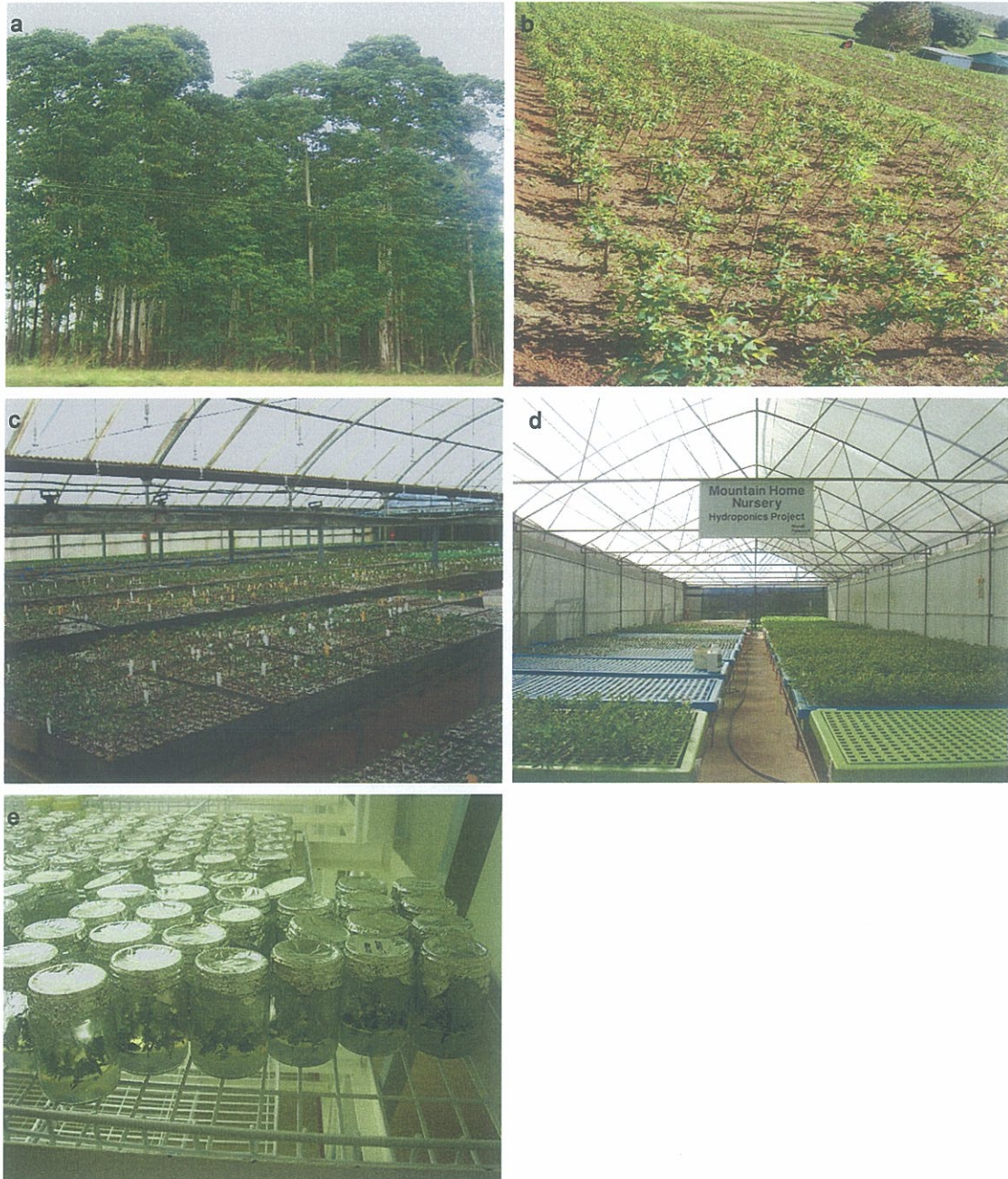


Figure 1.2: Examples of the methods of propagating *Eucalyptus* that are currently employed by Mondi Group, South Africa: (a) Field plantations- mature trees from which seeds can be collected; (b) clonal hedges; (c) cuttings maintained within the greenhouse; (d) cuttings grown on the hydroponic system; and (e) the culture of *in vitro* shoots on semi- solid nutrient medium.

1.3 Stresses Associated with Cryopreservation

There are a number of stresses that are associated with the preparation of plant tissue for cryopreservation and these are considered to be cumulative (Padayachee, *et al.*, 2008). These stresses occur from the moment the plant tissue is cultured *in vitro*. Light microscopy research conducted on banana showed that cryopreservation leads to the death of a vast number of highly vacuolated cells during the rapid freezing process (Helliot, *et al.*, 2003). There are few reasons for the death of these cells, *viz*:

- Damage caused during harvesting of the plant tissues for *in vitro* propagation and the handling of the small plant tissue during culturing.
- Physical dehydration (e.g. over activated silica gel) and chemical desiccation (use of cryoprotectants and encapsulation) are further stresses that the plant tissues is presented with in preparation for cryopreservation.
- Following cryo - preparation, the plant tissues is then placed in liquid nitrogen, where it is encounters additional stresses such as vitrification and freeze dehydration. These stresses can be detrimental to the survival of the plant tissues especially if the above culture preparation methods were ineffective.
- The regeneration of the plant tissues following cryostorage involves placing the tissue on hormone free nutrient medium. This leads to additional stress in the form of osmotic shock (the dehydrated plant tissues is placed on semi-solid nutrient medium) in the *in vitro* environment.

In order to ameliorate the impact of the combined effect of these stresses, it is important to consider each individually and understand their interactions.

1.3.1 Desiccation Stress

Plants may be classified as being desiccation tolerant if they can rehydrate successfully after intense desiccation without suffering permanent damage to the cell wall structures (Moore, *et al.*, 2008). Gene expression that may lead to desiccation tolerance is

considered to be triggered at the very onset of drying (Hoekstra *et al.*, 2001). A common hormone that is triggered is ABA (Moons, *et al.*, 1997 and Ma, *et al.*, 2006). The exogenous application of ABA also triggers the induction on genes that are only usually expressed during the dehydration process (Bartels and Salamini, 2001). Desiccation sensitive plants undergo a much greater degree of damage during desiccation, so much so that they generally do not recover post desiccation.

Apart from changes in gene expression, desiccation damage is expressed as changes in the cellular mechanisms e.g. carbohydrate metabolism, ultrastructural changes and perturbations of the photosynthesis related processes (Bartels and Salamini, 2001). The plant protective mechanism responsible for protecting and repairing the plant cells can also be damaged and this leads to a poor recovery post desiccation (Alpert, 2000). Desiccation cellular damage is inflicted both chemically and physically. Chemical damage can be caused by chemical compounds (such as the cryoprotectant solutions) and the production of free radicals that lead to oxidative damage within plant cells (Alpert, 2000). Physical damage mainly arises due to the mechanical stresses (e.g. handling and isolation of axillary buds) presented to the plant tissues and the response is common in *in vitro* storage techniques (Padayachee, *et al.*, 2008). Other physical damage such as turgor loss inflicts internal cellular damage of cells (Alpert, 2000) that may eventually lead to necrosis.

1.3.2 Salt stress

Plant growth is greatly affected by environmental stresses such as extreme temperatures, drought and even salinity. Globally there has been an increase in dryland salinity on agricultural productions, leading to a decrease in crop yield (Sun and Dickinson, 1993; Mansour and Salama, 2004). Many investigations have therefore been conducted to test the effects of salt tolerance on tree species. In particular, plant tissue culture techniques have been used to produce salt tolerant plants (Sweby, *et al.*, 1994).

When plants are subjected to high salinity, they undergo various physiological and biochemical changes. Salinity disrupts homeostasis in water potential and ion distribution, which can lead to molecular damage, growth arrest and even death (Zhu, 2001). Essentially an increase in salinity causes an increase in the cytosolic Ca^{2+} , Na^+ , and Cl^- ions that move from the apoplast to the vacuole where they are compartmentalized to prevent cytotoxicity within the plant cells (Hasegawa, *et al.*, 2000; Zhu, 2001; Yokoi, *et al.*, 2002). Cell membrane structure, enzyme activity, nutrient uptake and photosynthetic apparatus are all known to be affected by the toxicity of high salinity (Zhu, 2001). Reactive oxygen species (ROS) may be generated as damage occurs (Zhu, 2001; Shalata and Neumann, 2001). These damage essential membrane lipids as well as proteins and nucleic acids (Shalata and Neumann, 2001). In response, plants may produce osmolytes (Zhu, 2001) and antioxidants (Shalata and Neumann, 2001), which can detoxify plant cells/tissues by scavenging the ROS thus, preventing them from disrupting and damaging cellular structures.

Maintaining plant homeostasis is important when achieving tolerance to high salinity levels. The movement of salt into roots and shoots is usually due to the transpirational flux required to maintain the water potential of the plant (Hasegawa, *et al.*, 2000; Yokoi, *et al.*, 2002). Plants need to protect actively growing cells from salt ion accumulation. This is achieved by the movement of ions into cells (or cell compartments) of the tissues, usually the vacuole (Hasegawa, *et al.*, 2000; Zhu, 2001; Yokoi, *et al.*, 2002). During the uptake of NaCl , Na^+ competes with K^+ for intracellular influx because these cations are transported by common proteins (Hasegawa, *et al.*, 2000). It should be noted that K^+ is an important co- factor for enzymes whereas Na^+ is not; however, Na^+ is taken up because it is an important osmolyte in the vacuole (Hasegawa, *et al.*, 2000).

Chloride ions are known to interact with the levels of nitrogen uptake in plants (Grattan and Grieve, 1999). Elevated concentrations of Cl^- ions reduce the NO_3^- uptake by plants (Klobus, *et al.*, 1988; Kozłowski, 1997; Grattan and Grieve, 1999), and elevated concentrations of NO_3^- inhibits phosphate uptake (Kozłowski, 1997). However, if Ca^{2+} is applied exogenously, the uptake of NO_3^- under saline conditions increases (Klobus, *et al.*,

1988). According to Sweby, *et al.*, (1994), tobacco plant growth (that was treated with NaCl) *in vitro* was found to be superior when the plants were supplied with a NO_3^- source. Ammonium was shown to have an inhibitory effect on the growth of tobacco plantlets *in vitro*.

Numerous *Eucalyptus* species have been shown to tolerate high salinity levels (Blake, 1981; Morabito, *et al.*, 1994; Morabito, *et al.*, 1996) and, hence, would be ideal for afforestation in saline areas. Generally, the *Eucalyptus* species have been selected for their salt tolerance based on greenhouse experiments and experimental plots, which involved watering *Eucalyptus* trees with saline solutions. Sun and Dickinson (1993) examined the response to salt stress of 16 eucalypt species. *E. grandis* was shown to tolerate saline solutions up to 150mM NaCl. However, at a concentration of 200mM NaCl, this species died (Sun and Dickinson, 1993).

1.3.3 Cross stress tolerance

Plants encounter various environmental stresses that they need to tolerate. In order for them to combat these stresses, a series of events occurs that starts with the perception of the stress and ends up with the response elicited by a series of target genes (Knight and Knight, 2001; Pastori and Foyer, 2002). It has been observed that the plants defensive response to one stress may also allow for resistance to other stresses, this phenomenon is termed cross tolerance (Bowler and Fluhr, 2000). In addition 'cross- talk' is defined as: 'two signaling pathways from different stressors that converge' (Knight and Knight, 2001). 'Cross- talk' between two stress pathways might allow these pathways to achieve the same end response, or the pathways may interact with each other, thus affecting each others' ultimate outcome (Knight and Knight, 2001). It is this cross-talk that would allow a plant to become cross tolerant. Cross tolerance allows plants to make use of common pathways and components in response to biotic and/or abiotic stresses (Pastori and Foyer, 2002; Mundy, *et al.*, 2006). Work done on plant signaling has lead many scientists to believe that the linear pathway of a single plant response is only a small part of the more complex signaling network (Knight and Knight, 2001).

Although many investigations have been successful in producing or increasing salt tolerance in plants, minimal attention has been focused on cross stress tolerance e.g. the use of salt to improve the desiccation tolerance of plants.

1.4 Cryopreservation

Cryopreservation is defined as the storage of biological material at subzero temperatures usually at -196°C in liquid nitrogen (Panis and Lambardi, 2005; Benson, 2008). As a result of freezing, all metabolic processes cease, and the sample does not undergo any genetic alteration during storage (Withers, 1986; Fuller, 2004). Hence, cryopreservation is the ideal method of choice for the storage for specific genotypes (selected according to their desirable traits), and for the storage of unidentified genotypes for the purpose of maintaining a wide gene pool. Most plant material, however, cannot be stored at subzero temperatures without affecting the viability. This is due to the formation of ice crystals (intra- or extra-cellular) (Fuller, 2004) that cause damage to the cells and tissues (Panis and Lambardi, 2005). Consequently, the water content of the plant material is a crucial factor when considering plant material for long term cryostorage, and must be reduced via various pretreatment regimes in order to achieve success.

1.4.1 Methods of Cryopreservation

For cryopreservation to be successful, there are certain variables that need to be considered. These include; the size of the specimen to be cryostored, the water content of the specimen, the application of cryoprotectants (if necessary), the rate of freezing and thawing of the specimen, and the recovery environment (Mycock, *et al.*, 1997). There are several pretreatment strategies that plant materials can be subjected to for successful cryostorage, and these can be employed either as a single treatment or in combination.

1.4.2 Pretreatment Strategies

There are three pretreatment strategies in the cryopreservation of plant material.

- i. Culture pretreatment.
- ii. Chemical pretreatment.
- iii. Desiccation pretreatment.

i. Culture Pretreatment

Plant tissue culture entails regenerating plants from cells, tissues and pieces of organs in a sterile environment on artificial nutrient medium containing plant growth regulators, vitamins and a carbon source. The *in vitro* propagation of plant cells or tissues also inflicts stress on the explant by means of mechanical perturbation, injuries, wounding, desiccation (through exposure to air), and osmotic shock caused by different forms of nutrient medium (e.g high sucrose concentrations, altered ions or hormonal adjustments) and the infiltration of the culture medium into the intracellular air spaces (Gasper, *et al.*, 2002).

Macronutrients included in the tissue culture medium are: nitrogen, calcium, potassium, phosphorus, magnesium and sulphur. Micronutrients are found in smaller quantities and include iron, chlorine, manganese, zinc, boron copper, molybdenum and occasionally nickel. Organic supplements, usually in the form of vitamin B (myo-insitol and thiamine) are often added to the culture medium (George, 1993), as they are considered beneficial for the culture of plant cells *in vitro*. Other vitamins that may be added are nicotinic acid, pyroxine (George, 1993).

Additives to plant tissue culture media other than the fundamental nutrients can assist in the preparation of biological material for cryopreservation. These can include plant growth regulators (PGR's) which trigger specific biological responses that enable drying (Padayachee, 2000) or osmotically active substances, e.g. sucrose and polyethelene glycol (PEG), which can remove cellular water in a gradual and presumably biologically regulated manner (Panis and Lambardi, 2005).

Sugars in Preparation for Cryopreservation

The carbohydrate source that is added to *in vitro* culture medium is a sugar, such as sucrose. There are various other forms of sugars that can be used in *in vitro* culture e.g. sorbitol, glucose, maltose or galactose. As sucrose is a cheap carbon source and is easily taken up by the plant, it is most commonly used. However, as pointed out by George (1993) sucrose in the culture medium specifically inhibits chlorophyll formation and photosynthesis, and hence affects autotrophic growth. The addition of sucrose to the growth media also affects the osmotic potential of the medium, and with increasing concentrations of sucrose, the water content within plant cells decreases (George, 1993). Thus, the greater the sucrose concentration, the lower the water content, and the greater the possibility of the explants suitability for cryopreservation.

Plant Growth Regulators (PGR's)

Plant growth regulators include auxins, cytokinins, gibberellins, abscisic acid and ethylene and they have different functions in morphogenesis and cryopreservation (Table 1).

Absciscic acid

Absciscic acid (ABA) plays a crucial role in plant responses to environmental stresses, where it serves as a link between the stress factors and the plant response (Skriver and Mundy, 1990; Swamy and Smith, 1999; Wang, *et al.*, 2002). Responses to environmental stresses including cold, drought and high salinity are mediated by ABA with numerous plant species showing an increase in endogenous ABA concentrations (Swamy and Smith, 1999; Wang, *et al.*, 2002). The presence of ABA, whether endogenous or exogenously applied, results in the expression of a number of genes and dehydration related proteins (Wang, *et al.*, 2002).

Table 1: Summary of roles of the common plant growth regulators in tissue culture and cryopreservation.

Hormone	Natural Function	Role in <i>in vitro</i> Culture	Role in Cryopreservation
Auxins	Cell division and cell growth in roots. Role in apical dominance. Common auxins are Indoleacetic acid (IAA) and Indol-3-butanoic acid (IBA)	Cell division and cell growth in roots. IAA is the common auxin used in plant tissue culture.	Support recovery growth after controlled freezing. Possible role in shoot and callus production.
Cytokinins	Influence cell division and shoot formation. Responsible of mediating auxin transport in plants. Cytokinins are often associated with the development of photosynthetic apparatus in plant cells (Arigita, et al., 2005)	Responsible for shoot formation <i>in vitro</i> . Can be responsible for callus formation in tissue culture, depending on the form of cytokinins used. Common cytokinin: benzyl aminopurine (BAP)	Commonly used in the recovery medium of cryostored plant cells as it aids in shoot proliferation.
Ethylene	Affects fruit ripening and plays a role in senescence. Naturally produced by the plant in the form of a gas (Kende and Zeevaart, 1997).	Ethylene is regarded as a stress hormone (Konishi and Yanagisawa, 2005). Ethylene may accumulate in the culture vesicle and thus affect the growth and development of the plant.	Can regulate the accumulation of antifreeze proteins in response to cold and drought stress (Yu, Griffith and Wiseman, 2001)
Gibberellins	Role in seed germination and can promote cell elongation.	Involved in regulating cell elongation and is important in determining plant height and fruit production.	Together with auxins, gibberellins aid in recovery and growth following freezing
Absciscic Acid	Produced in leaves and acts as an inhibitory compound that affects bud growth (section below for more detail)	The exogenous application of ABA is important as it assists the plant in plant water homeostasis.	Used to prepare the plant for cryopreservation by assisting in lowering the water content of the tissue to be cryostored.

The expression of ABA responsive genes varies according to the type of and degree of stress in plant tissues (Skriver and Mundy, 1990). Late Embryogenesis Abundant (LEA) proteins have been associated with increasing desiccation tolerance in seeds. LEA proteins are regulated by osmotic stress caused by drought, salinity, and cold (Xiong and Zhu, 2002; Tunnacliffe and Wise, 2007), and have been proposed to function as protein and membrane stabilizers, antioxidants, ion sinks and hydration buffers during water stress (Wise and Tunnacliffe, 2004; Tunnacliffe, 2008).

Absciscic acid is synthesized *de novo* in response to drought and high salinity stress. Many stress inducible genes are regulated by the endogenous ABA that accumulates during high salinity (Shinozaki and Yamaguchi-Shinozaki, 2000; Shinozaki, *et al.*, 2003; Xiong and Zhu, 2003) and this results in the accumulation of stress gene products. The stress gene products include various kinds of proteins and smaller molecules such as sugars, proline and glycine betaine (Shinozaki and Yamaguchi-Shinozaki, 2000). ABA is not the only product produced in response to environmental stress. Other products include reactive oxygen species and calcium. The production of these compounds all react differently and do not all participate in the same reactions i.e. some will respond to drought and some will respond to salinity, with or without the presence of ABA. It is, therefore, likely that both ABA-independent and ABA-dependent signal cascades exist (Shinozaki and Yamaguchi-Shinozaki, 2000; Shinozaki, *et al.*, 2003; Xiong and Zhu, 2003), and since many of the same genes respond to drought and salinity, the existence of ‘crosstalk’ between the signaling pathways is likely.

ii. Chemical pretreatment

Tissues may be desiccated chemically through the use of high concentrations of osmotically active substances, often referred to as cryoprotectants. The term cryoprotectant can be defined as “an additive which can be provided to cells before freezing and yields a higher post-thaw survival than can be obtained in its absence” (Fuller, 2004). Cryoprotectants are a heterogeneous collection of compounds, which decrease the freezing and super-cooling point of water (Katha, 1985; George, 1993), by dehydrating and penetrating into the cells. Cryoprotectants can be classified as penetrating (e.g. glycerol) or non-penetrating (sucrose) (Mycock, *et al.*, 2004) and are usually most effective when used in combination. Penetrating cryoprotectants include glycerol and dimethylsulfoxide (DMSO). These two substances are characterized by having low molecular weights and the ability to penetrate cells rapidly, hence creating an osmotic gradient that allows the glycerol and the DMSO to be deposited in plant cells and tissues (Wilkins and Dodds, 1983). This allows glycerol and DMSO to act as protective

substances to the nucleic acid, mitochondria and the membranes of plant cells (Wilkins and Dodds, 1983).

Non-penetrating cryoprotectants, such as sucrose act by causing osmotic dehydration and thus reduce the water content of hydrated plant tissues (Dumet, *et al.*, 1993). Sucrose also acts by stabilizing membranes and proteins during desiccation, and is a common component of cryoprotectant solutions (Dumet, *et al.*, 1993).

As there are certain cryoprotectants that can be poisonous to the plant material (e.g. DMSO) it is essential to determine their correct combination and concentration (Mycock, *et al.*, 2004). Padayachee (2000) examined the effects of long term storage of encapsulated *E. grandis* axillary buds that were subjected to the step-wise cryoprotection with sucrose and DMSO. These proved to be non toxic to the axillary buds compared with the range of cryoprotectants that were tested (Padayachee, 2000).

iii. Desiccation pretreatment

The type of explant used for cryopreservation is an important factor as the water content differs in the various parts/tissues of the plant. It is recommended that the smaller and within the limits, the drier the explant, the better (Karthi, 1985; Baek, *et al.*, 2003; Mycock, *et al.*, 2004). The rate of water loss from plant tissues and the water content to which the material is dried are critically important factors (Mycock, *et al.*, 2004) for cryopreservation. Meristems are the most appropriate plant organs to use for cryopreservation as they are genetically stable (unlike calli and cells in suspension). However, meristems are generally highly hydrated and, therefore, need to be dried to lower water contents that are appropriate for cryopreservation.

During freezing, most of the protoplasmic water is lost and very low amounts of tightly bound water remains in the cell (Phillips, *et al.*, 2002). Most vegetative tissues of plants are sensitive to such extreme water loss and this is manifested as extensive cellular damage (Benson, 1994). The extent of damage may be reduced by the methods of artificial drying that are chosen. There are several such methods, e.g. drying over

activated silica gel (Stewart, *et al.*, 2001) and drying in the air flow of a laminar hood (Mycock, 1999; Panis and Lambardi, 2005), with the former being the preferred method of in the present investigations. The physical drying of the tissue using activated silica gel has been used to desiccate numerous species for successful cryopreservation (e.g. Dumet, *et al.*, 1993).

The exposure time of plant tissue to the dry environment created by the silica gel varies between species. Generally, the longer the drying period, the drier the sample. However, plants can only be dried for certain periods, after which the vigour and viability of the material becomes compromised (Mycock, *et al.*, 2004). Padayachee (2000) showed that *E. grandis* axillary buds that were desiccated for longer than 20 minutes over activated silica gel exhibited reduced viability. Similarly, Stewart *et al.* (2001) who were determining the methods for the cryostorage of Cassava somatic embryos showed that the desiccation of embryos over silica gel for 20 minutes resulted in the reduction of the water content while maintaining the viability of the embryos.

1.4.3 Freezing

The size of the sample is very important as this factor controls the rate of freezing, with less ice crystals forming within smaller samples. There are two methods of freezing that can be used for the cryopreservation of plant material namely, slow freezing and rapid freezing.

- Slow freezing involves the controlled rate or stepwise reduction in temperature to sub zero temperatures. It allows tissues to be further dehydrated as water is drawn out of the cells to an area where extracellular ice nucleation occurs (Benson, 1994; Benson, 2008).
- The rapid freezing approach involves the immersion of the explant into a cryogen, usually liquid nitrogen, and in this case, the rate of freezing is approximately -200°C per minute. The rapid transfer of the explant through the point of ice nucleation prevents ice formation i.e. there is minimal intracellular ice development (Meryman and Williams, 1985; Lambardi, *et al.*, 2005).

Both methods are effective in cryopreserving plant tissue as intracellular injury is reduced. Following cryogen storage (the period of which can range up to decades), the explants are then thawed.

1.4.4 Thawing

Rapid thawing is mostly used to maintain the viability of the plant tissue. At -130°C vitreous ice structures can become crystalline and at -70°C ice crystals can nucleate from existing ice crystal structures (Meryman and Williams, 1985). Rapid thawing can be achieved by plunging the cryovials (containing the material) into sterile distilled water at 25-30°C. Thereafter, the thawed *in vitro* plant material is plated on nutrient medium for recovery.

1.4.5. Recovery Growth

The re-growth of plants is a sensitive test as it represents the performance of plant cells following the various stresses to which they have been exposed. Following cryopreservation, the thawed explants are usually placed on hormone free nutrient medium for recovery. The nutrient medium is usually hormone free to allow plant to adapt to the change in environment, thus not shocking the cell with increased levels of hormones.

1.5. AIM

The aim of this project was two fold: firstly, the determination of the range of NaCl concentrations that *E. grandis in vitro* axillary buds could tolerate without the loss of vigour and viability, and secondly, to determine whether cross stress tolerance to desiccation was induced by exposure to NaCl, thus allowing for the successful cryostorage of *E. grandis* axillary buds, by the ability of the tissue to drop to lower water contents.

2. MATERIALS AND METHODS

2.1 Plant materials and culture conditions

Mondi Group, South Africa, provided *Eucalyptus grandis* (clone ZG14) rooted cuttings (Figure 2.1). These were transplanted into Culterra Potting Soil®. The plants were maintained in a greenhouse throughout the project. They were treated with three fertilizers (10ml.l⁻¹ Trelmix, 1g.l⁻¹ Mondi Orange and 0.3g.l⁻¹ Calmag) and two fungicides. The one fungicide mixture, containing 1ml.l⁻¹ Bravo and 2g.l⁻¹ Dithane, was applied as a foliar spray, along with the fertilizers. The second fungicide mixture (systemic fungicide), containing 1.25ml.l⁻¹ Folicur and 1ml.l⁻¹ Sporgon was applied to the soil. The *E. grandis* plants were alternatively treated weekly, with one fungicide and one fertilizer.

2.2 *In vitro* shoot production

Following six weeks of fertilizer and fungicide treatment, *E. grandis* shoots containing new lateral branches were harvested and surface decontaminated in 0.2g.l⁻¹ mercuric chloride for 10 minutes followed by three washes in water. Thereafter, the shoots were excised into nodal cuttings and rinsed thoroughly with sterile distilled water (Millipore Milli Q) followed by a second decontamination with 10g.l⁻¹ calcium hypochlorite for 10 minutes. The nodal trimmings were once again rinsed in sterile distilled water and further decontaminated using a mixture of 50mg.l⁻¹ penicillin and 50mg.l⁻¹ streptomycin for 10 minutes. The decontaminated material was cultured on semi-solid multiplication medium comprising MS salts (Murashige and Skoog, 1962), 0.2mg.l⁻¹ BAP, 0.01mg.l⁻¹ NAA, 0.1mg.l⁻¹ biotin, 0.1mg.l⁻¹ calcium panthothenate, 25g.l⁻¹ sucrose and 3g.l⁻¹ Gelrite (Figure 2.2). The pH of the nutrient medium was adjusted to 5.7 before autoclaving (SA-300VF ®) for 20 minutes.

Nodal cuttings from the plants established in the greenhouse were surface decontaminated (as above) and established *in vitro* cultures. *E. grandis in vitro* shoot

clusters were maintained in the growth chamber at 25°C under a 16h light photoperiod at $200\mu\text{E}^{-2}\text{s}^{-1}$ photosynthetic photon flux density. The subculture of material was conducted every 4- 6 weeks by transfer onto fresh semi solid multiplication nutrient medium in Magenta jars (400ml). The multiplied shoots were then transferred to elongation medium containing MS salts (Murashige and Skoog, 1962), 0.1mg.l^{-1} calcium panthothenate, 0.35mg.l^{-1} NAA, 0.1mg.l^{-1} IBA, 25mg.l^{-1} sucrose and 3g.l^{-1} Gelrite (pH of 5.7) for 2 weeks. The axillary buds from these shoots were used in all experiments.



Figure 2.1: An *E. grandis* (clone ZG14) plant that was maintained in the greenhouse at the University of Witwatersrand.



Figure 2.2: Nodal explant that had been established *in vitro* (± 4 weeks) on multiplication medium (also known as bud break medium)

2.3 Stress Treatment

2.3.1 Experimental Design

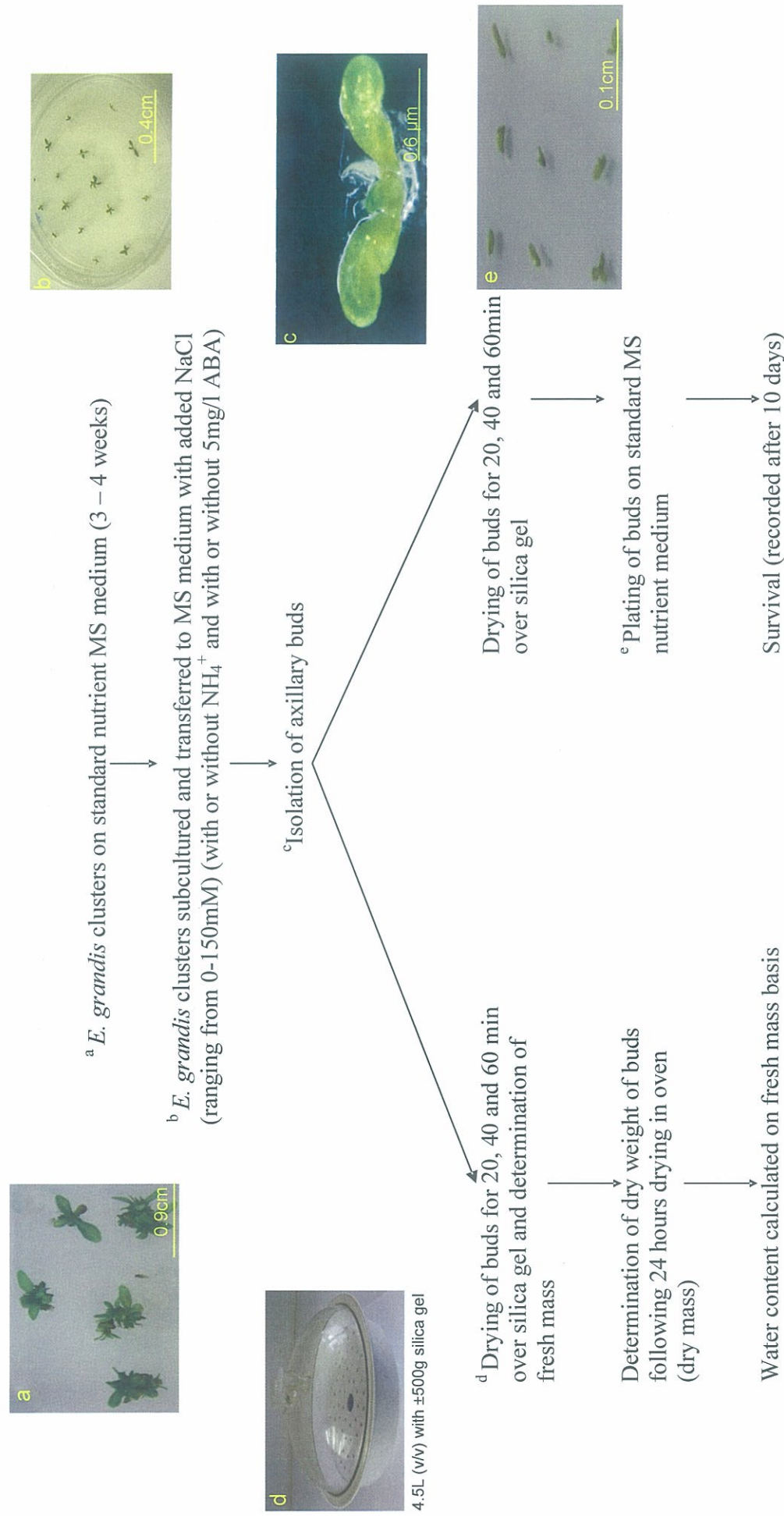


Figure 2.3: Experimental design of the protocol for the induction of cross-stress tolerance of *E. grandis* by using NaCl, with or without the addition of NH_4^+ to the nutrient medium.

2.3.2 Sodium chloride treatments

Clusters were subjected to three treatments (Figure 2.3):

- i. Shoots cultured on sodium chloride (NaCl) supplemented culture medium for 14 days
- ii. Shoots cultured on culture medium containing NaCl and ABA in the culture medium for 14 days
- iii. Shoots treated with ABA in the culture medium for 5 days, followed by NaCl for 14 days.

Shoot clusters (approximately 3-5 shoots per cluster) were cultured in Petri dishes (90mm) containing multiplication medium and various concentrations of NaCl. Pilot experiments were conducted to determine the range of NaCl that *E. grandis* would be able to survive, and at which concentrations the *E. grandis* clusters displayed signs of injury. The initial NaCl concentrations ranged between 0- 400mM. Results obtained indicated that concentrations of 150mM NaCl and above, for 14 days, resulted in severe stress, initially in the form of leaf browning, and later in the form of necrosis. These were undertaken with the standard nutrient MS formulation (containing the full concentration of nitrogen in the form of NH_4^+ and NO_3^-), and the modified MS with only NO_3^- (only 40mM of nitrogen in the form of nitrate), as well as plant growth regulators, agar, and sucrose.

Consequently subsequent experiments employed a concentration range of 0- 150mM NaCl. Clusters were cultured on MS medium (with or without NH_4^+) containing NaCl for 14 days and the degree of injury was noted. Thereafter, the axillary buds were isolated and the water content and survival was assessed by means of weight determination and visual observations respectively. Figure 2.3 demonstrates the schematic representation of the experimental procedure.

Furthermore, because of the alteration of nitrogen levels in the nutrient medium, several controls were conducted. These included:

- i. 16.37g/ l of K_2SO_4 and 19 g/l of KNO_3 i.e. equivalent levels of potassium as that added to make up 40mM of NO_3^- in the form of KNO_3 .

- ii. 19g/l of KNO₃ i.e. 20mM of nitrogen in the form of NO₃⁻, (normal concentration of potassium but only half the concentration of nitrate, as in standard MS medium).
- iii. 27.75g/l of (NH₄)₂SO₄ i.e. 20mM of nitrogen in the form of NH₄⁺ in the nutrient medium (equivalent to that found in MS nutrient medium).
- iv. 16.37 g/l of K₂SO₄ + 27.75 g/l of (NH₄)₂SO₄ i.e. normal levels of potassium and NH₄⁺ and no NO₃⁻.

2.3.3 ABA treatment

In order to establish the effect of ABA on water loss from the axillary buds, only selected NaCl concentrations were chosen. A low, medium and high NaCl concentration were considered sufficient.

The pretreatment involving ABA was conducted in one of two ways.

- i. Shoot clusters were initially cultured on multiplication medium (with and without NH₄⁺) containing NaCl (i.e. 0, 1, 20, 100mM NaCl) with 5mg. l⁻¹ ABA for 14 days and the degree of injury was noted. The axillary buds were excised, and the water content and survival was assessed (Figure 2.3), before and after drying over silica gel.
- ii. Shoot clusters were cultured on 5mg. l⁻¹ ABA for five days, followed by 14 days on nutrient MS medium (with and without NH₄⁺) and NaCl (i.e. 0, 1, 20, 100mM NaCl), thereafter, the degree of injury was recorded. The axillary buds were excised and the water content and survival was assessed (Figure 2.3), before and after drying over silica gel.

Two controls were used for these experiments viz *E. grandis* clusters on 5mg. l⁻¹ ABA for five days and *E. grandis* clusters on 5mg. l⁻¹ ABA for 14 days. The degree of injury was noted following the allocated time frame (either 5 days or 14 days) and thereafter, the water content and viability was determined.

2.4 Data Collection

2.4.1 Desiccation using silica gel and water content determination

E. grandis axillary buds (n=20) were dehydrated in a desiccator (4.5l v/v), over activated silica gel (± 500 g). The silica gel was stabilized to 25°C, 24 hrs prior to the desiccation procedure. The buds were placed on foil pans inside the desiccator (Figure 2.4) and dried for 20, 40, and 60 minutes, after which the water content of the buds was gravimetrically determined.



Figure 2.4: Desiccator containing activated silica gel (4.5L (v/v) with ± 500 g silica gel)

To determine the water content of hydrated *E. grandis* axillary buds, the fresh mass of twenty individual buds was determined. Buds were dried in an oven at 80 °C for 24 hrs, and the dry mass of the buds was recorded. The difference between the fresh and dry weight was determined and expressed as a percent of the fresh weight (wet weight basis).

2.4.2 Assessment of injury and survival

Survival was determined by the ability of *in vitro* *E. grandis* axillary buds to survive (i.e. remain green for 10 days) the salt stress with or without desiccation on standard and modified nutrient MS medium (i.e. full concentration of nitrogen in the form of NH_4^+ and NO_3^-). The degree of injury to *E. grandis* clusters prior to desiccation was assessed by means of visual observations, based on the following scaling system: 0 = no injury; 1 = leaves or shoot browning; 2 = necrosis; and 3 = death (Figure 2.5). The survival of the axillary buds was monitored for 10 days following the stress treatments.

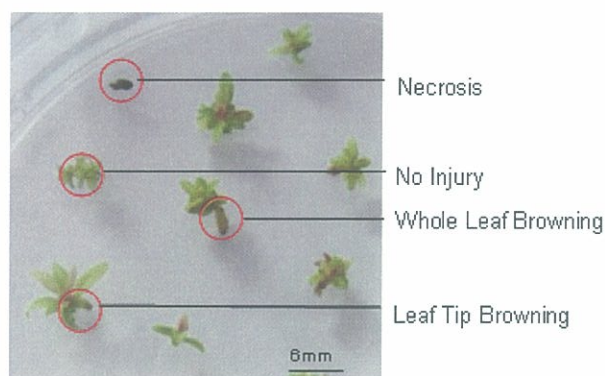


Figure 2.5: Section of a Petri dish Indicating levels of observable injury.

2.4.3 Replicates and Statistical Analysis

All experiments were replicated three times. Averages and standard errors were calculated for water content (expressed as a percentage on the fresh weight basis) and survival assessments. One Way Analysis of Variance (ANOVA) was used (at a 95% confidence level) to assess any significant differences between the water content at different salt concentrations, and the different pretreatment strategies. The statistical analysis, according to SAS, assigned alphabetical values to the mean values recorded per treatment. Mean values that do not share the same letter were recognized as being significantly different.

3. RESULTS

3.1 Physiological effects of culturing *E. grandis* on standard MS medium (40mM N- NO₃⁻ and 20mM N- NH₄⁺) supplemented with various concentrations of NaCl

Cluster gross morphology: The growth response of *E. grandis* clusters that were placed on standard nutrient medium (containing 40mM NO₃⁻ and 20mM NH₄⁺) (Murashige and Skoog, 1962) with or without NaCl is shown in Figures 3.1 and 3.2. The negative effect of the presence of NaCl in the growth medium was found to be directly related to the concentration of NaCl supplied. With increasing NaCl concentrations, there was an increase in the injury exhibited by the *E. grandis* clusters. Leaf tip browning and whole leaf browning increased as the concentration of NaCl increased, thus indicating that NaCl inflicted a certain amount of stress on the *E. grandis* clusters (Figure 3.1). When comparing the results for leaf tip browning with the control, there was a 15% increase in the number of clusters that were injured and, between the control and 10mM NaCl, there was a 23% increase in the number of clusters that experienced leaf tip browning (Figure 3.1). Similarly, between the control and 10mM NaCl treatment, there was an 18% increase in the number of clusters that experienced whole leaf browning whilst between the control and 100mM, there is 23% increase in whole leaf browning (Figure 3.1). It was also evident that as the NaCl concentration increased, necrosis increased. Between 50mM NaCl to 100mM NaCl, there was an increase in the injury expressed by the *E. grandis* clusters (Figure 3.1).

It should be noted that even for the control, there was a certain percentage of clusters that exhibited signs of leaf tip browning, whole leaf browning and necrosis, i.e senescence due to the stressful culture conditions and handling during subculturing.

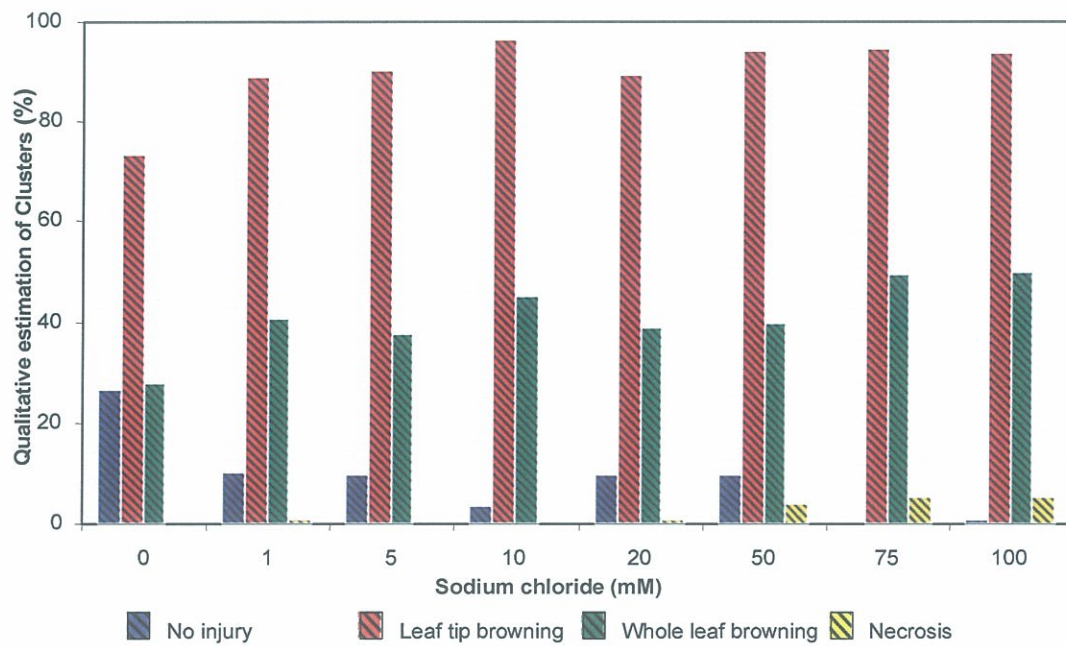


Figure 3.1: The injury caused to *E. grandis* clusters that were cultured on standard MS medium with various concentrations of NaCl for 14 days. The injury is expressed as a percentage of the total number of clusters that were cultured on the nutrient medium (n=20).

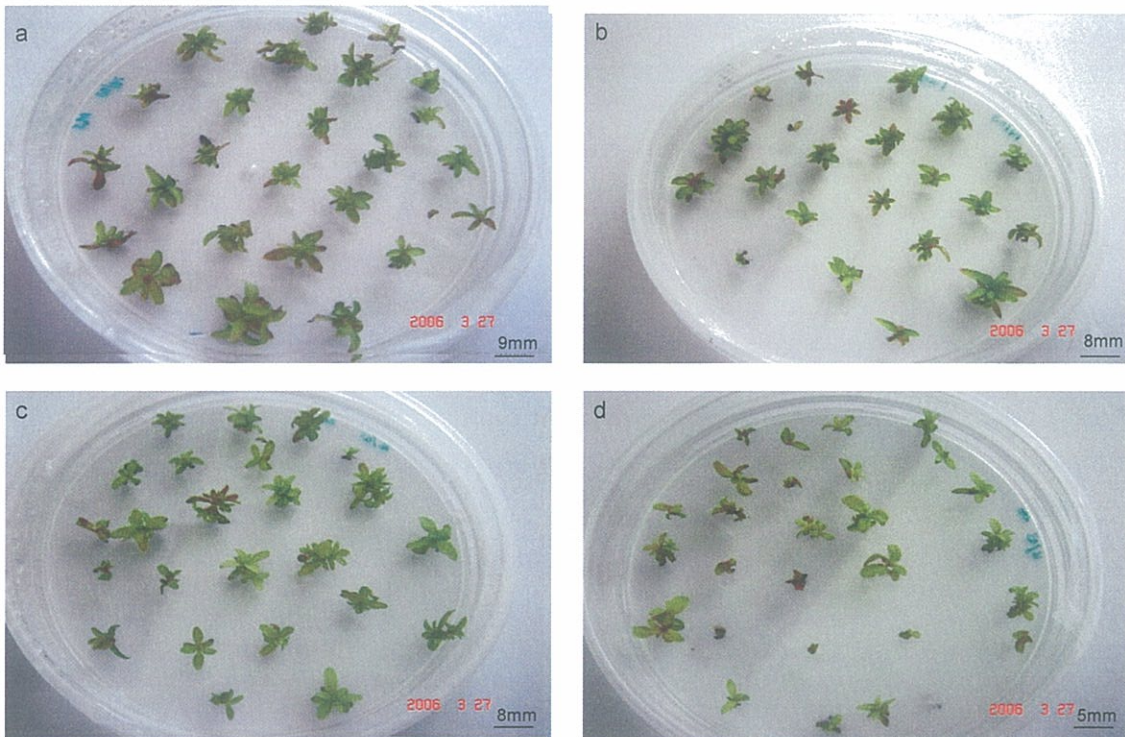


Figure 3.2: *E. grandis* clusters that were cultured on standard nutrient MS medium with NaCl (a) MS only (b) MS + 1mM NaCl (c) MS + 20mM NaCl (d) MS + 100mM NaCl.

The effect of desiccation on *in vitro* axillary buds, isolated from clusters that had been cultured on standard nutrient MS medium in the presence of NaCl: The ability of *E. grandis* axillary buds to lose water and survive desiccation can be observed in Figure 3.3. The water content (represented as the line graphs) ranged from $65\% \pm 1.61$ (no drying) to $40\% \pm 1.82$ (after 60 minutes drying), with a decline in viability from 100% (after no drying) to an average of $11\% \pm 1.66$ after (60 minutes of drying) (Figure 3.3). This indicated that the addition of NaCl to the nutrient medium did not positively affect the water content (i.e. lower the water content) of the axillary buds without negatively impacting the survival of the buds. The survival of *E. grandis* axillary buds decreased by more than 50% after being desiccated for 20, 40, and 60 minutes (Figure 3.3).

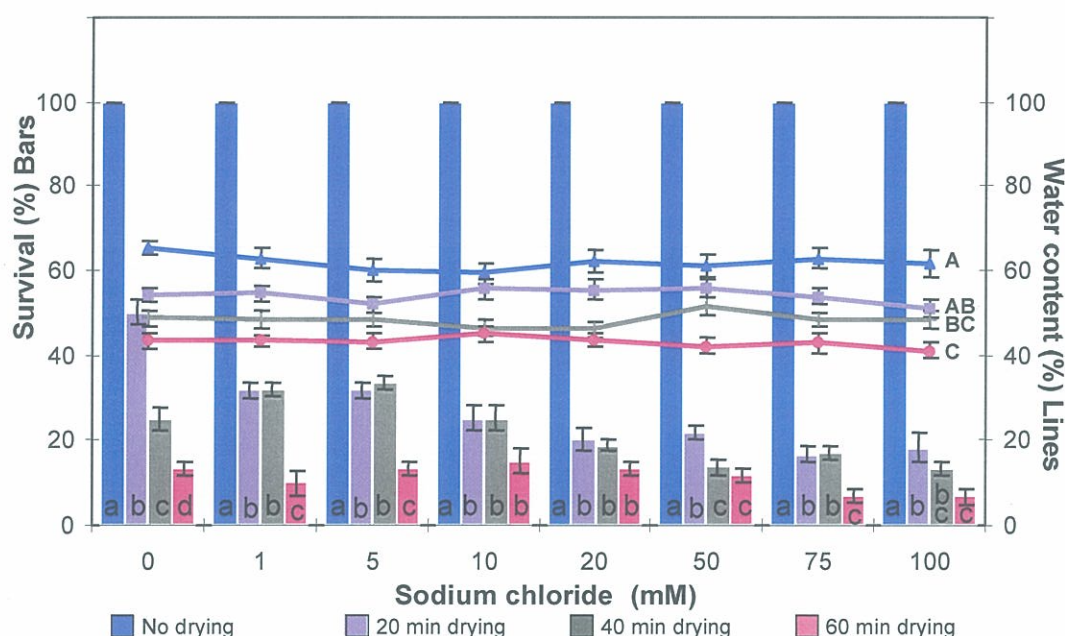


Figure 3.3: The effect of NaCl on the percentage survival (indicated as bars) and the percentage water content (indicated as lines) of isolated axillary buds, over a 60 minute drying treatment. The buds were cultured in standard MS medium and NaCl for 14 days, thereafter, the mean water content (expressed as a percentage of the fresh weight) and the viability (expressed as percentage survival of treated buds after 10 days on standard MS medium) were determined. Bars indicate standard errors and the different letters (a-d) indicate significant differences; $n=20$; $p < 0.0001$ for water content and survival.

3.2 Physiological effects of culturing *in vitro* *E. grandis* axillary on modified MS medium (40mM N- NO₃⁻) supplemented with various concentrations of NaCl

The investigation that was performed in section 3.1 utilised the full concentration of nitrogen the Murashige and Skoog (1962) medium (40mM NO₃⁻ and 20mM NH₄⁺), with the addition of NaCl. In the present investigation, the nutrient medium only contained two thirds (i.e. 40mM NO₃⁻-N) the amount of nitrogen as that found in Murashige and Skoog (1962) nutrient medium i.e. no N- NH₄⁺ was added. The elimination of NH₄⁺ from the nutrient medium was performed in order to manipulate the plant into taking up N- NO₃ instead of Cl⁻ thereby preventing the toxic ionic effects of Cl⁻(see earlier).

Cluster gross morphology: The growth response of *E. grandis* clusters (before the isolation of axillary buds) that were placed on modified MS nutrient medium (containing 40mM NO₃⁻ only) (Murashige and Skoog, 1962) with or without NaCl may be observed in Figure 3.4 and Figure 3.5. With increasing concentration of NaCl and in the absence of NH₄⁺, the number of explants that demonstrated no injury decreased, and the number of explants that experienced leaf tip browning, whole leaf browning, and necrosis increased (Figure 3.4). The 10mM NaCl concentration had the highest number of explants that had leaf tip browning (a 30% increase over the control), and, beyond that concentration, the number of explants that died increased. An endogenous bacterial contamination was expressed at the higher NaCl concentration. The NaCl exposed explants also appeared to be paler green, thus suggesting an effect on the photosynthetic abilities of the clusters (Figure. 3.5). The removal of NH₄⁺ plus the addition of NaCl stressed the plant to levels where whole leaf browning and necrosis increased.

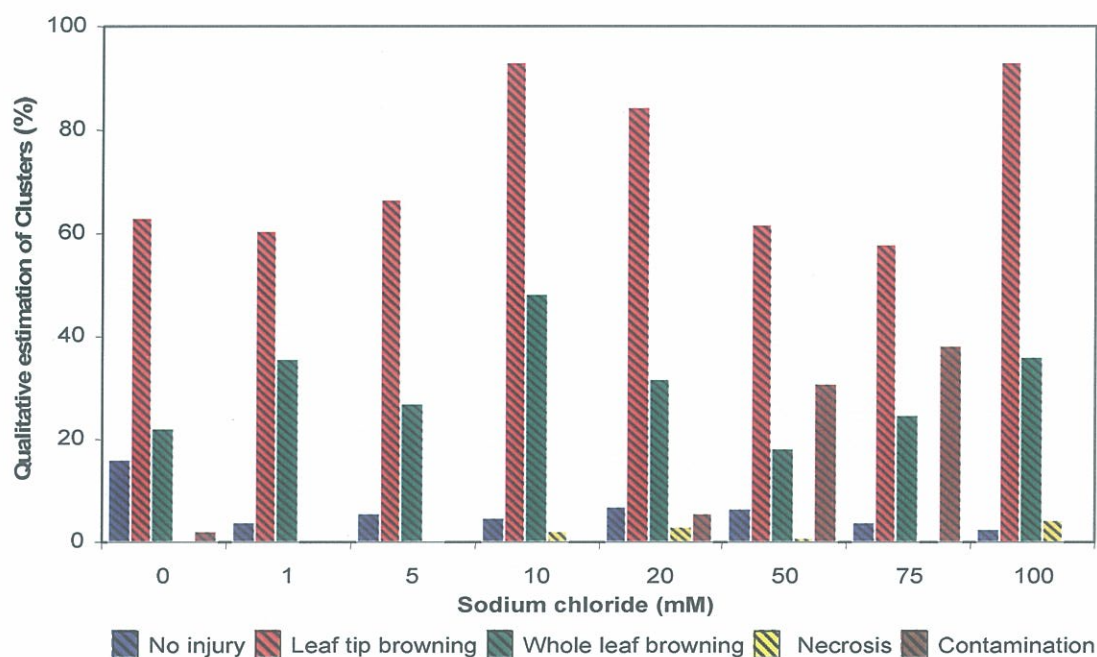


Figure 3.4: The injury caused to *E. grandis* clusters that were cultured on modified MS medium (40mM NO_3^-) with various concentrations of NaCl for 14 days. The injury is expressed as a percentage of the total number of clusters that were cultured on the nutrient medium.

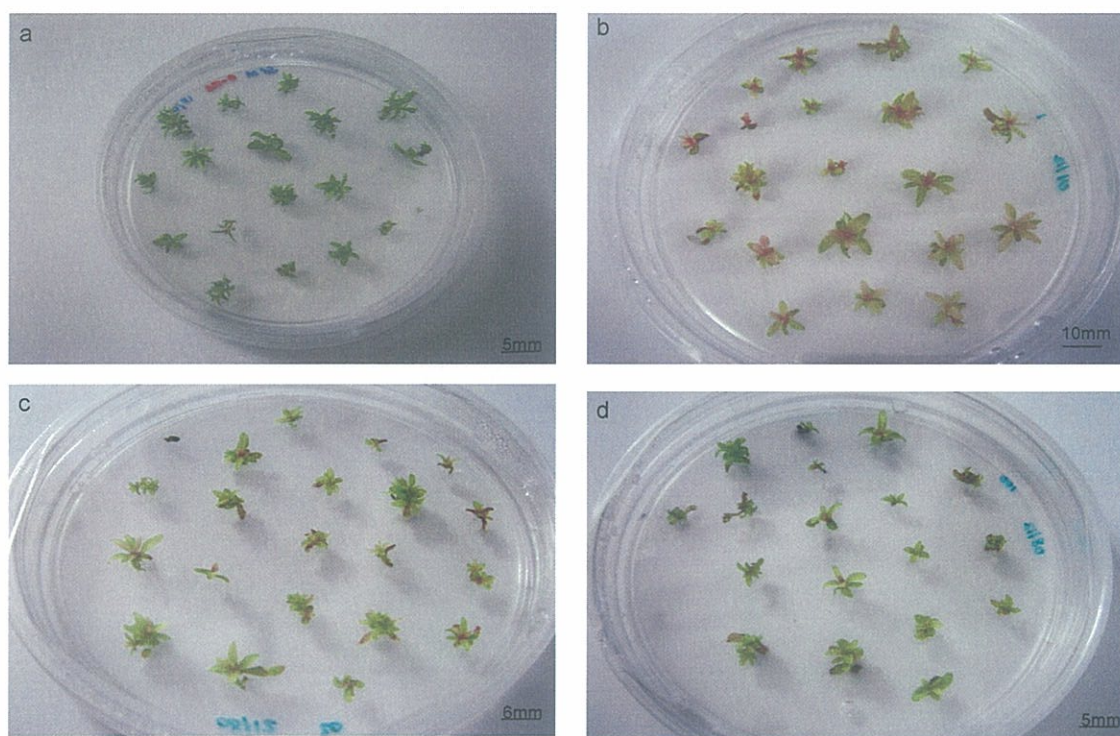


Figure 3.5: *E. grandis* clusters that were cultured on modified nutrient MS medium (40mM NO_3^-) with NaCl (a) 40mM NO_3^- only (b) 40mM NO_3^- + 1mM NaCl (c) 40mM NO_3^- + 20mM NaCl (d) 40mM NO_3^- + 100mM NaCl.

The effect of desiccation on *in vitro* axillary buds, isolated from clusters that had been cultured on modified nutrient MS medium (40mM N- NO₃⁻) in the presence of NaCl:

The ability of *E. grandis* axillary buds to lose water and survive desiccation can be observed in Figure 3.6. The water content of the buds ranged from 62% ± 1.82 (no drying) to 44% ± 1.59 (after 60 minute drying). There was no significant difference between the NaCl concentrations of the same drying time for no drying, 20 and 40 minutes. However, 60 minutes of drying showed a significant difference (95% confidence level) between the control, 75 and 100mM to 1, 5, 10, 20 and 50mM NaCl. The results showed that the addition of NaCl to the nutrient medium and the removal of NH₄⁺ did not allow for the material to be dried below 44% without compromising viability. However, drying reduced the viability of the material and 60 minutes of desiccation lowered the survival to below 10%. At the lower concentrations of NaCl, there was an increase in the number of clusters that showed leaf tip browning and necrosis (i.e. 10mM NaCl), as compared with the nutrient medium that contained the full concentration of nitrogen in the form of NO₃⁻ and NH₄⁺ (compare Figure 3.1 and Figure 3.4). The axillary buds cultured on the modified nutrient MS medium also had a lower survival rate than those cultured in the presence of NO₃⁻ and NH₄⁺. After 20 minutes of desiccation, there was approximately 70% loss of survival on the modified MS nutrient MS medium, (without NaCl), compared with a 50% loss in survival on the standard nutrient MS medium (without NaCl). The amount of water loss from the *E. grandis* axillary buds was greater (but not significantly different) when cultured in the nutrient MS medium that contained the full concentration of nitrogen: water content ranged between 62% ± 1.82 to 40% ± 1.82, compared with the modified MS nutrient medium where the water content ranged between 62% ± 1.82 to 44% ± 1.59 (Figure 3.3 and Figure 3.6). Survival of *E. grandis* axillary buds that were cultured on 40mM NO₃⁻ with the addition of NaCl, indicated that with increasing NaCl concentrations, the viability of the buds decreased. Thus in summary, the reduction in the overall nitrogen concentration and the elimination of NH₄⁺ from the nutrient MS medium, led to an increase in the number of clusters that demonstrated injury and loss of viability.

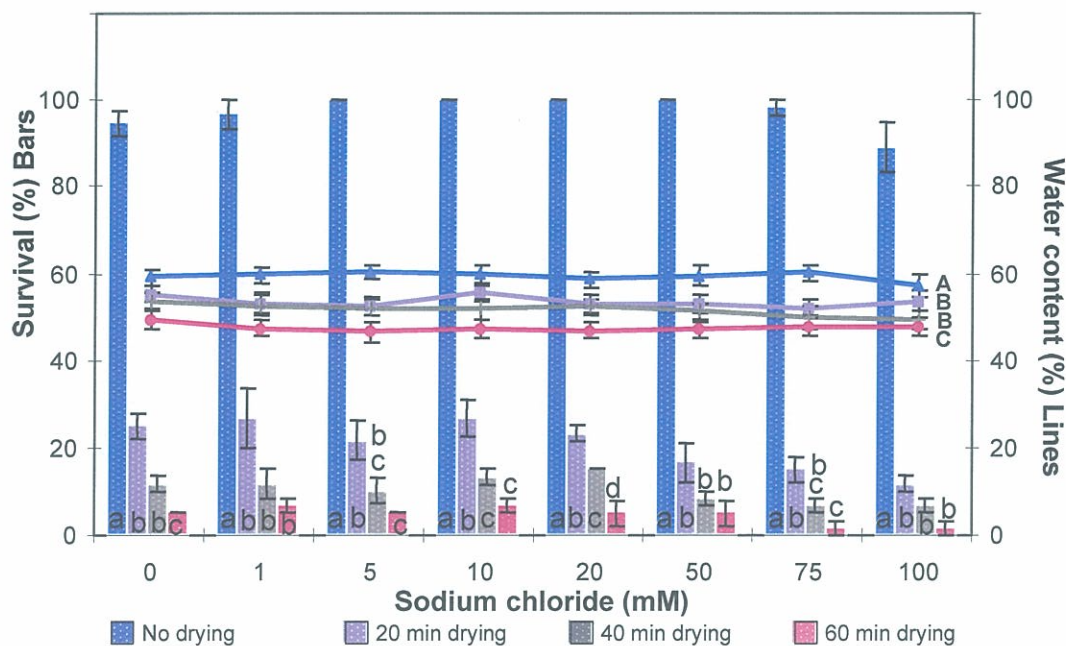


Figure 3.6: The effect of the concentrations of NaCl on the growth medium on the percentage survival (indicated as bars) and the percentage water content (indicated as lines) of isolated axillary buds, over a 60min drying treatment. The buds were cultured in modified MS medium (40mM NO_3^-) and NaCl for 14 days, thereafter, the mean water content (expressed as a percentage of the fresh weight) and the viability (expressed as percentage survival of treated buds after 10 days on standard MS medium) was determined. Bars indicate standard errors and the different letters (a-d) indicate significant differences; $n=20$, $p < 0.0001$ for survival only.

In order to maintain the nitrogen levels in the modified medium equivalent to that found in Murashige and Skoog (1962) it was necessary to increase the amount of KNO_3 in the medium. However, this also altered the K^+ levels in the medium and this necessitated the investigation of several other control media.

There were four parameters that were investigated:

- Doubled concentration of K^+ (supplied as K_2SO_4), and full concentration of NO_3^- : this experiment was conducted to investigate the effect of elevated levels of potassium in the nutrient medium i.e. 40mM NO_3^- (supplied as KNO_3) + K_2SO_4
- Normal concentration of NH_4^+ (supplied as $(\text{NH}_4)_2\text{SO}_4$) and K^+ (supplied as K_2SO_4): this experiment was conducted to test the toxicity of NH_4^+ when provided as the sole nitrogen source.

- Normal concentration of K^+ and half the concentration of NO_3^- : this parameter was investigated to determine if any of the physiological responses demonstrated by *E. grandis* *in vitro* explants were due to the uptake of K^+ .
- Standard MS concentrations of NH_4^+ but no NO_3^- and no K^+ : this nutrient medium contained the same concentration of NH_4^+ as that found in nutrient MS and was used as a control.

The response of the various treatments on *E. grandis* clusters may be observed on Figure 3.7. The *E. grandis* clusters that had been cultured on nutrient medium whose sole nitrogen source was NO_3^- , showed signs of leaf reddening (Figure 3.7a & c). These clusters also appeared to have fewer shoots and smaller leaves (but this was not quantified). In comparison, clusters whose sole nitrogen source was NH_4^+ showed more leaf tip and whole leaf browning (Figure 3.7b and d), presumably due to NH_4^+ toxicity.

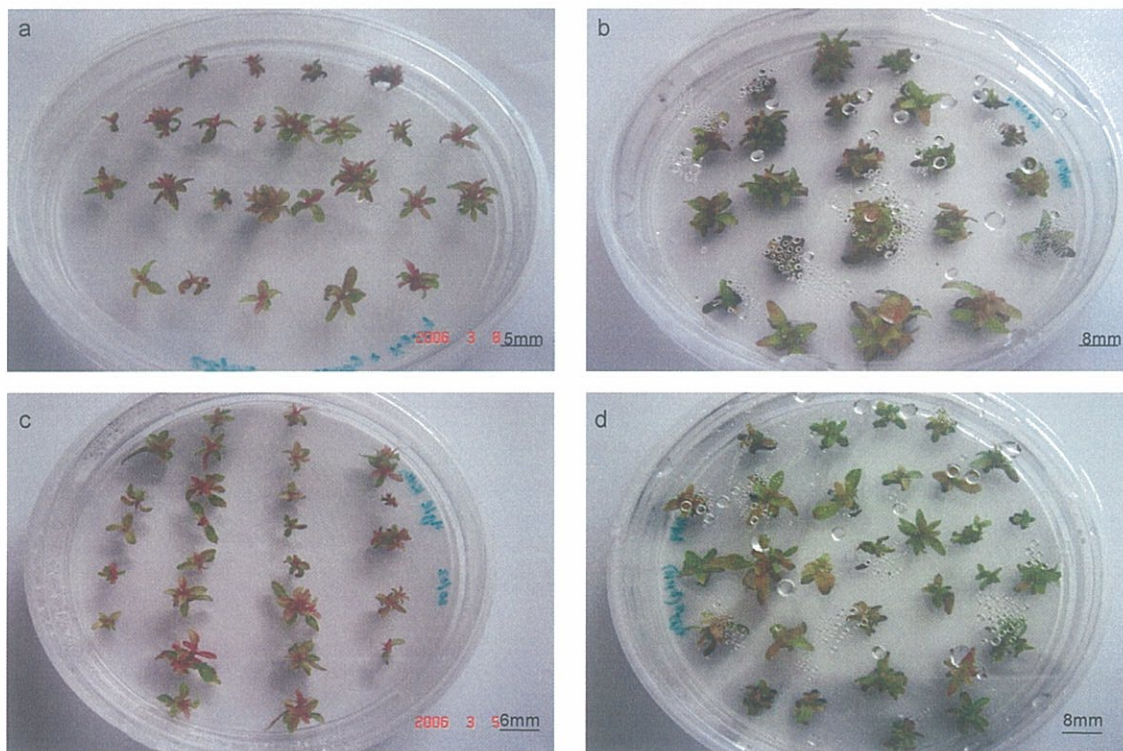


Figure 3.7: *E. grandis* clusters that have been cultured on modified MS formulations (a) $KNO_3 + K_2SO_4$ (b) $K_2SO_4 + (NH_4)_2SO_4$ (c) 20mM KNO_3 (d) $(NH_4)_2SO_4$.

The effect of desiccation on axillary buds, isolated from clusters that were cultured on different modified MS formulations: The response of *E. grandis* axillary buds to desiccation after being cultured on one of the four modified MS nutrient medium is displayed in Figure 3.8. The material that had been cultured on media that contained elevated levels of potassium ($\text{KNO}_3 + \text{K}_2\text{SO}_4$), did not dry to water contents lower than 46%. Statistically, there was no significant difference between the water content of the non- dried and 20 minute dried buds, and, 40 and 60 minute dried buds (Figure 3.8- line graph). There was approximately a 75% reduction in the survival of the axillary buds from $\text{KNO}_3 + \text{K}_2\text{SO}_4$ when they were subsequently desiccated. A similar trend was observed with material dried from the nutrient medium that contained normal levels of K^+ and half the amount of NO_3^- (20mM NO_3^- in the form of KNO_3). Again, the water content of the desiccated axillary buds did not drop lower than $46\% \pm 1.70$, and there was a 75% loss of survival of the desiccated axillary buds.

The material taken from the nutrient medium that contained NH_4^+ as the only nitrogen supply had water content values ranging between $64\% \pm 2.05$ (no drying) and $40\% \pm 2.48$ (after 60 minutes of drying), and, there was no survival of the axillary buds after 40 and 60 minutes of desiccation. However, the material from the medium that contained normal concentrations of K^+ and normal concentration of NH_4^+ (as that found in standard MS) showed a minor improvement in terms of water loss (Figure 3.8- line graph). The water content ranged between $68\% \pm 1.56$ (after no drying), and $37\% \pm 2.56$ (after 60 minutes of drying), similar to those buds that were isolated from clusters cultured on the standard MS nutrient medium without NaCl. There was no survival of these axillary buds after 60 minutes of desiccation (Figure 3.8)

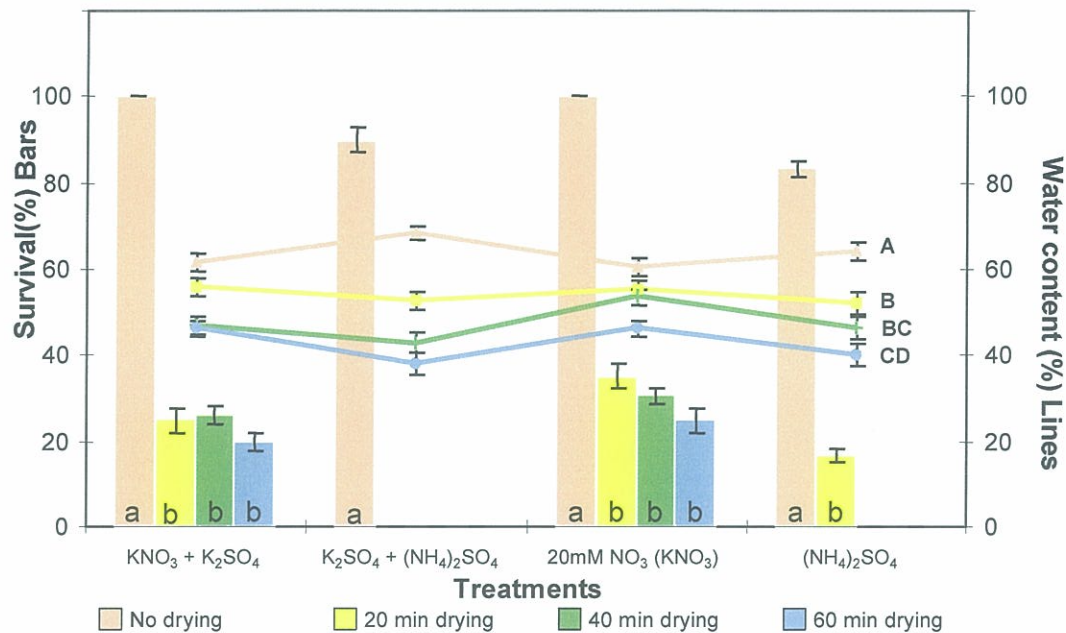


Figure 3.8: The drying response (lines) and survival (bars) of *E. grandis* axillary buds isolated from clusters that had been cultured on modified MS formulations. The mean water content is expressed as a percentage of the fresh weight. Bars indicate standard errors and the different letters (a-b) indicate significant differences; n=20, p <0.0001 for water content and survival.

Collectively the data showed that the *E. grandis* axillary buds retained a high water content and low survival, regardless of nutrient medium on which the clusters had been cultured.

3.3. Physiological effect of ABA on NaCl stressed *E. grandis* explants.

Cluster gross morphology: The growth response of *E. grandis* clusters that were cultured on ABA for either 5 or 14 days may be observed in Figure 3.9 and Figure 3.10. After the 5 day treatment, almost 80% of the clusters displayed signs of whole leaf browning and approximately 5% of the clusters had died (Figure 3.9). More than 80% of the clusters that were cultured on ABA for 14 days displayed leaf tip browning, whole leaf browning, and phenolic production (Figure 3.10 b) and approximately 15% of the *E. grandis* clusters had died (Figure 3.9 and Figure 3.10 b). This indicated that the presence of ABA in the nutrient medium for longer than 5 days had a negative effect on *E. grandis* clusters.

The effect of desiccation on *in vitro* axillary buds, isolated from clusters cultured on ABA for either 5 or 14 days: There appeared to be no significant difference in water content between the axillary buds isolated from clusters that were cultured on ABA for 5 days or ABA for 14 days. The average water content ranged between 59 ± 2.35 to $53\% \pm 2.04$ and 58 ± 1.58 to $52\% \pm 2.07$ respectively (Figure 3.11). The survival of *E. grandis* axillary buds that were isolated from clusters cultured on ABA for 5 days decreased by 60% when desiccated for 20 minutes over silica gel. In contrast, *E. grandis* axillary buds that were isolated from clusters cultured on ABA for 14 days had a significantly lower survival rate than those cultured on ABA for 5 days (Figure 3.11). The application of ABA to the clusters imposed a certain amount of stress, and if ABA was continuously applied for longer than 5 days (as in the above scenario), the survival of the isolated axillary buds was reduced nearly 5-fold.

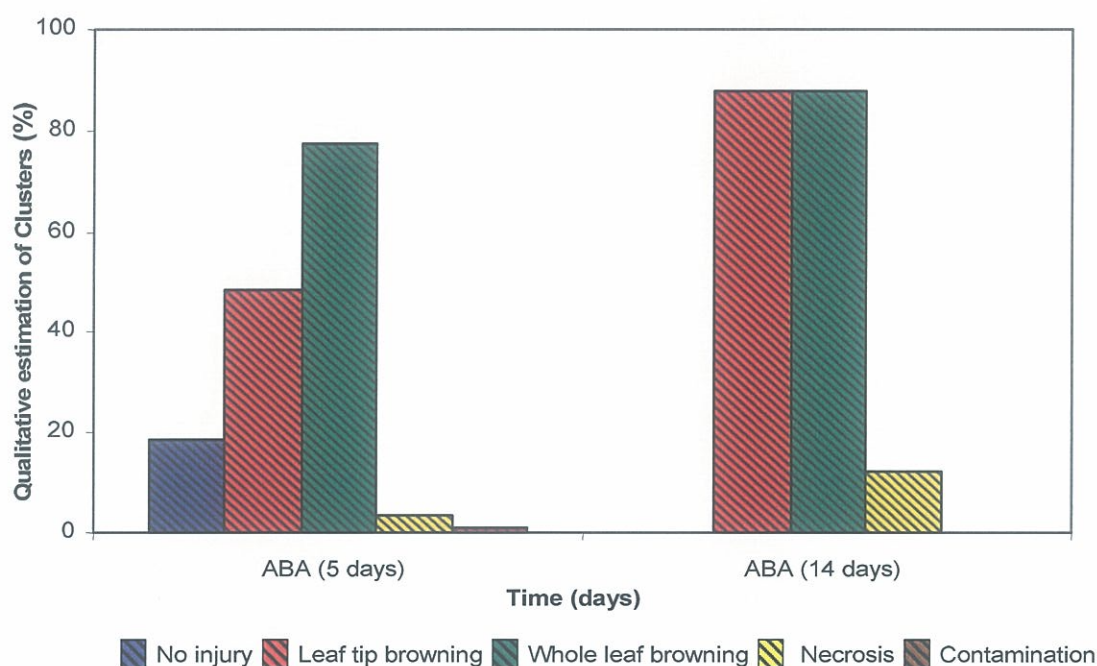


Figure 3.9: The degree of injury caused to *E. grandis* clusters that were cultured on ABA for 5 or 14 days. The degree of injury is expressed as a percentage of the total number of clusters cultured on the nutrient MS medium.

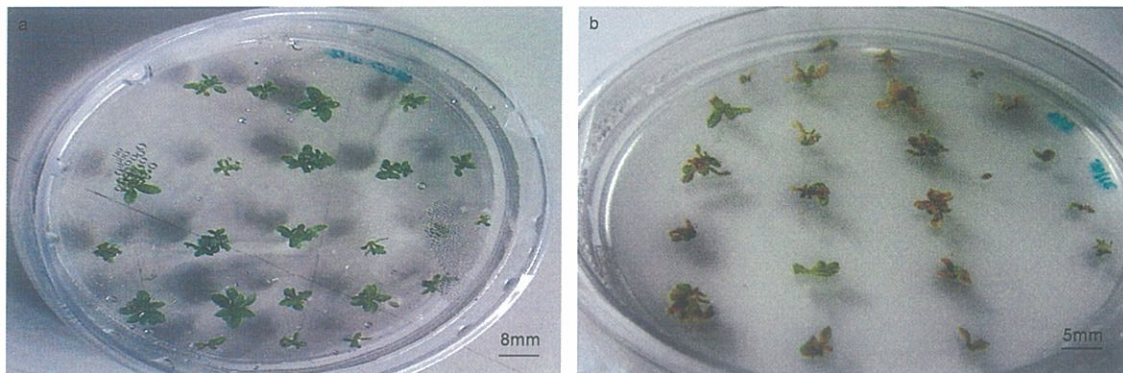


Figure 3.10: *E. grandis* clusters that have been cultured on (a) ABA for 5 days and (b) ABA for 14 days. Note the release of tannins/ polyphenolics from the 14 day culture

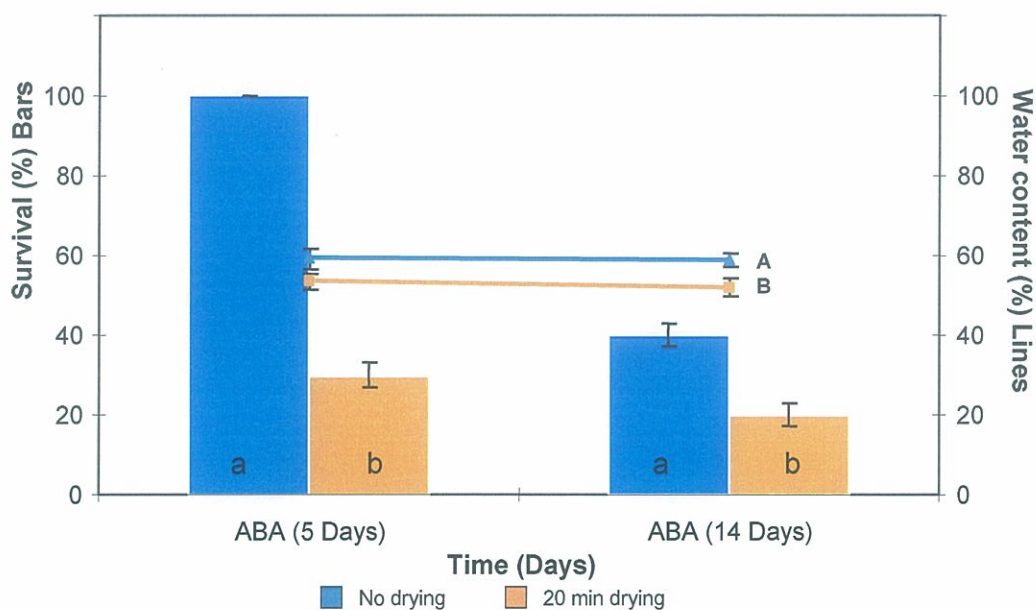


Figure 3.11: The drying response (lines) and survival (bars) of *E. grandis* axillary buds that were isolated from clusters and cultured on ABA for either 5 or 14 days without the addition of NaCl. The mean water content is expressed as a percentage of the fresh weight and the survival (expressed as percentage survival of the treated buds after 10 days on standard nutrient MS medium). Bars indicate standard errors and different letters indicate significant differences; $n=20$; $p < 0.0001$ (for viability) and $p = 0.0651$ (water content of buds on ABA for 5 days) and $p = 0.0149$ (water content of buds on ABA for 14 days).

Cluster gross morphology of *E. grandis* cultured on ABA supplemented media for 5 days prior to transfer to media containing NaCl:

E. grandis clusters that were cultured on standard MS nutrient medium containing 5mg/l ABA for a 5 day pre- treatment showed an increase in the number of clusters that did not display any injury. After transfer to NaCl containing media, it was observed that as the concentration of NaCl increased the number of explants that died decreased (Figure 3.12). At 1mM NaCl, the incidence of leaf tip browning decreased and whole leaf browning increased (Figure 3.12), thus indicating that the 5 day pretreatment on ABA did have some protective mechanisms on the *E. grandis* clusters. It is possible that the ABA assisted in the improving the desiccation tolerance and thereby the viability of the *E. grandis* clusters that were cultured on NaCl.

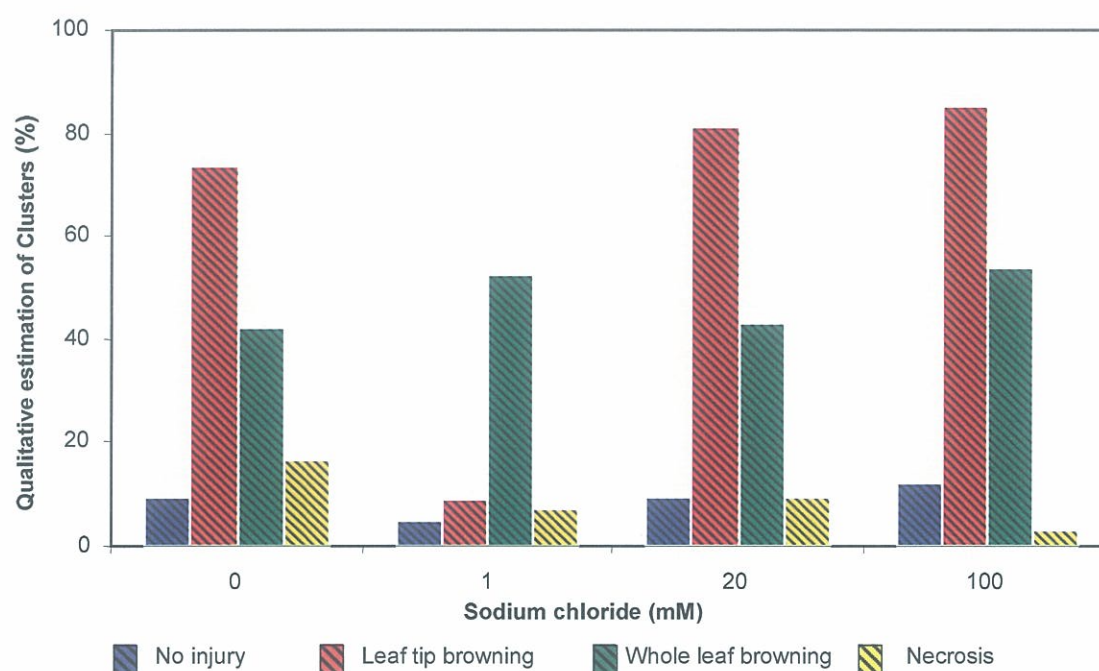


Figure 3.12: The degree of injury caused to *E. grandis* clusters that were cultured on ABA for 5 days, and thereafter on unmodified nutrient MS medium, together with various concentrations of NaCl for 14 days. The degree of injury is expressed as a percentage of the total number of explants cultured on the nutrient MS medium.

As the concentration of NaCl increased, the size of the clusters became smaller and this could possibly suggest that the addition of NaCl had a negative or inhibitory effect on the growth of *E. grandis* clusters (Figure 3.13a-d). There was an association between necrosis and the NaCl concentration: as the concentration increased, necrosis decreased (Figure 3.13). The cause for the high necrosis at 0mM NaCl was due to the small size of the clusters that were originally plated onto the ABA medium; these buds did not survive the initial 5 day ABA pre-treatment due to condensation in the Petri dish which caused flooding on the nutrient medium.

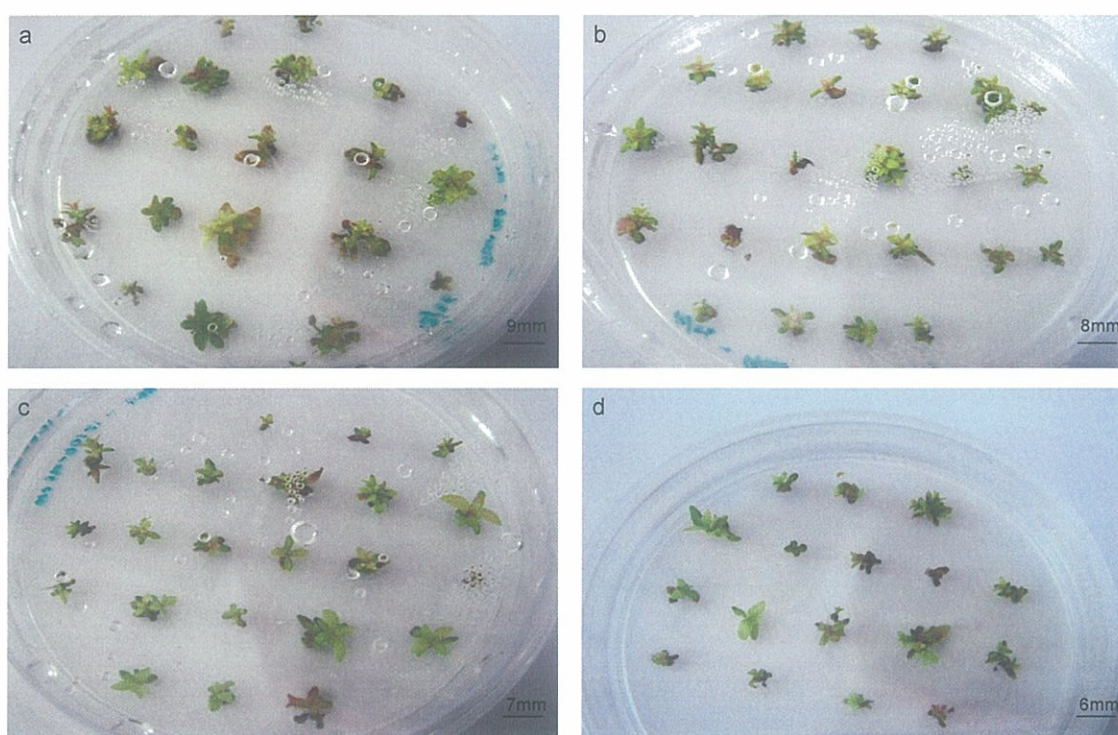


Figure 3.13: *E. grandis* clusters that were cultured on ABA for 5 days, and thereafter on standard nutrient MS medium with NaCl (a) MS only (b) MS + 1mM NaCl (c) MS + 20mM NaCl (d) MS + 100mM NaCl.

The effect of desiccation on *in vitro* axillary buds, isolated from clusters that were cultured on a 5 day ABA pre- treatment followed by a 14 day culture on standard MS medium with NaCl:
The water content of buds that were pre-cultured on ABA for 5 days and thereafter on nutrient MS medium with added NaCl for 14 days, ranged between $59\% \pm 2.38$ (no drying) to $45\% \pm$

1.68 (20 minutes of drying) (Figure 3.14). Furthermore, there was no significant difference across the drying times treatments (Figure 3.14).

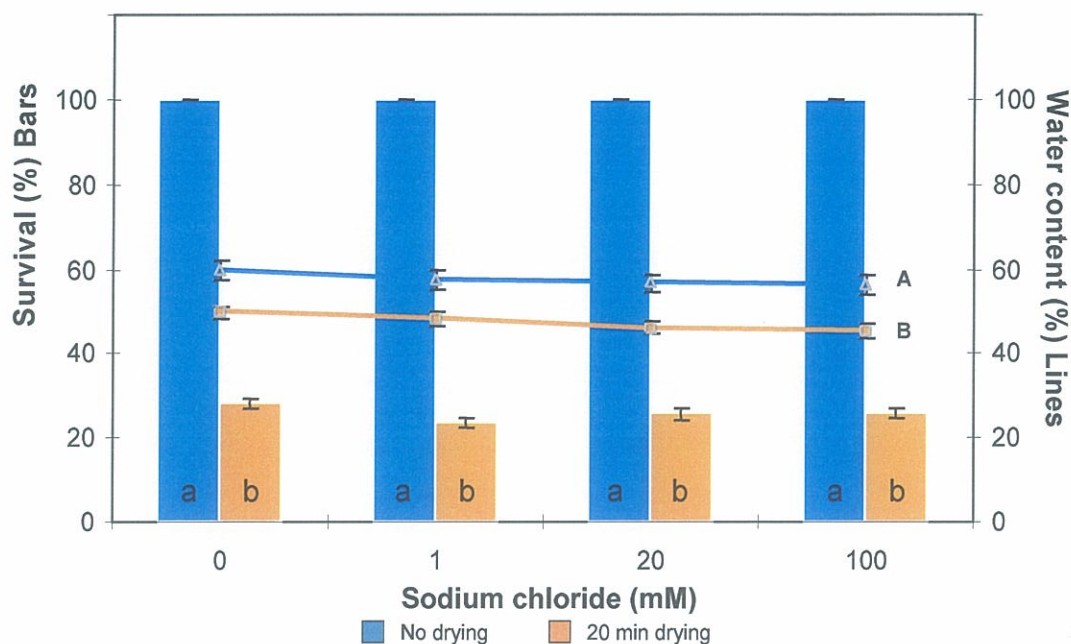


Figure 3.14: The drying (20 minutes over silica gel) response (lines) and survival of *E. grandis* axillary buds that were cultured on ABA for 5 days, and thereafter on standard MS media containing various concentrations of NaCl for 14 days. The mean water content is expressed as a percentage of the fresh weight, and the survival (expressed as percentage survival of the treated buds after 10 days on standard nutrient MS medium). Bars indicate standard errors and the different letters (a-b) indicate significant differences; n=20; p < 0.0001 (for survival) and p= 0.0006 (for water content).

The survival of buds that were pretreated on ABA for 5 days and thereafter on nutrient MS with added NaCl for 14 days ranged from 100% (with no drying), to 23% ± 1.20 (after 20 minutes of drying).

Cluster gross morphology of *E. grandis* cultured on modified MS nutrient medium (N-NO₃⁻) and NaCl, after a 5 day pre- treatment on ABA:

The number of clusters that demonstrated no signs of injury was minimal (Figure 3.15) when cultured for 5 days on modified nutrient MS (NO₃⁻) containing ABA. However, as the concentration of NaCl increased, the level of necrosis increased (Figure 3.15).

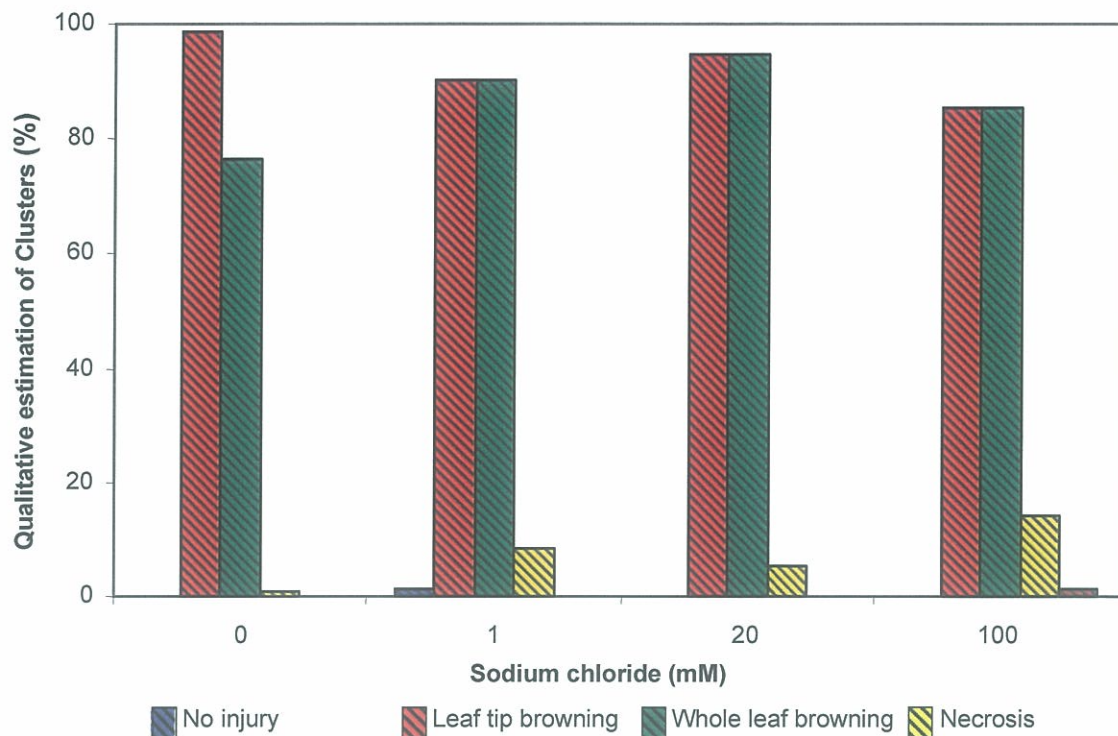


Figure 3.15: The degree of injury caused to *E. grandis* clusters that were cultured on ABA for 5 days, and thereafter on modified nutrient MS medium (40mM NO_3^-) together with various concentrations of NaCl for 14 days. The degree of injury is expressed as a percentage of the total number of clusters cultured on the modified nutrient MS medium.

Figure 3.16 displays the morphology of the *E. grandis* clusters after a 5 day ABA pre- treatment and 14 days on the modified nutrient MS medium (only NO_3^-) containing NaCl. Based on the qualitative data collected, the clusters decreased in size as the concentration of NaCl increased. There was also an increase in necrosis and at 100mM NaCl (Figure 3.16d); the leaves of the clusters appeared to be brittle and dry. As the concentration of NaCl increased, the number of clusters displaying whole leaf browning and necrosis increased.

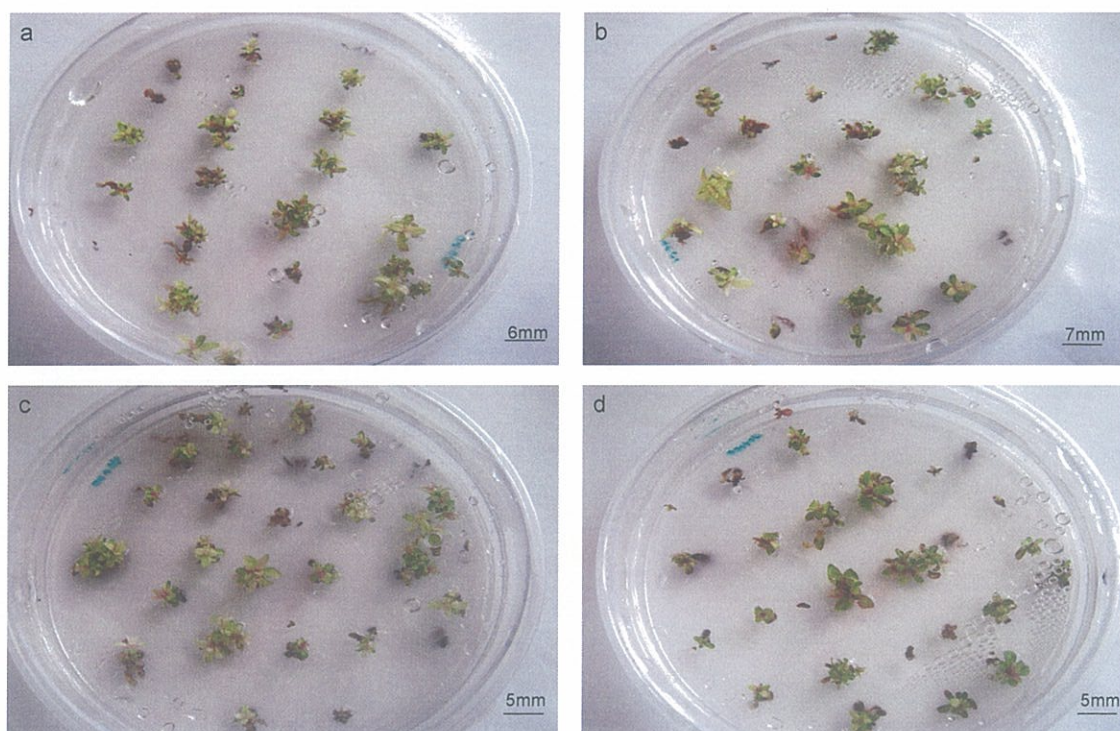


Figure 3.16: *E. grandis* clusters that were cultured on ABA for 5 days, and thereafter on modified nutrient MS medium (40mM NO_3^-) with NaCl (a) 40mM NO_3^- only (b) 40mM NO_3^- + 1mM NaCl (c) 40mM NO_3^- + 20mM NaCl (d) 40mM NO_3^- + 100mM NaCl.

The effect of desiccation on the *in vitro* *E. grandis* axillary buds, isolated from clusters that had been cultured on a 5 day ABA pre- treatment and thereafter on modified nutrient MS medium (40mM NO_3^-) with NaCl:

The presence of NaCl in the nutrient medium (regardless of the concentration) had no significant effect on the water content of the material (Figure 3.17). A similar trend was observed for material that had been dried for 20 minutes: the water content ranged between $58\% \pm 1.91$ (with no drying) and $46\% \pm 2.00$ (after 20 minutes drying, Figure 3.17). That was despite the 5 day ABA pretreatment, the *E. grandis* axillary buds maintained relatively high water contents.

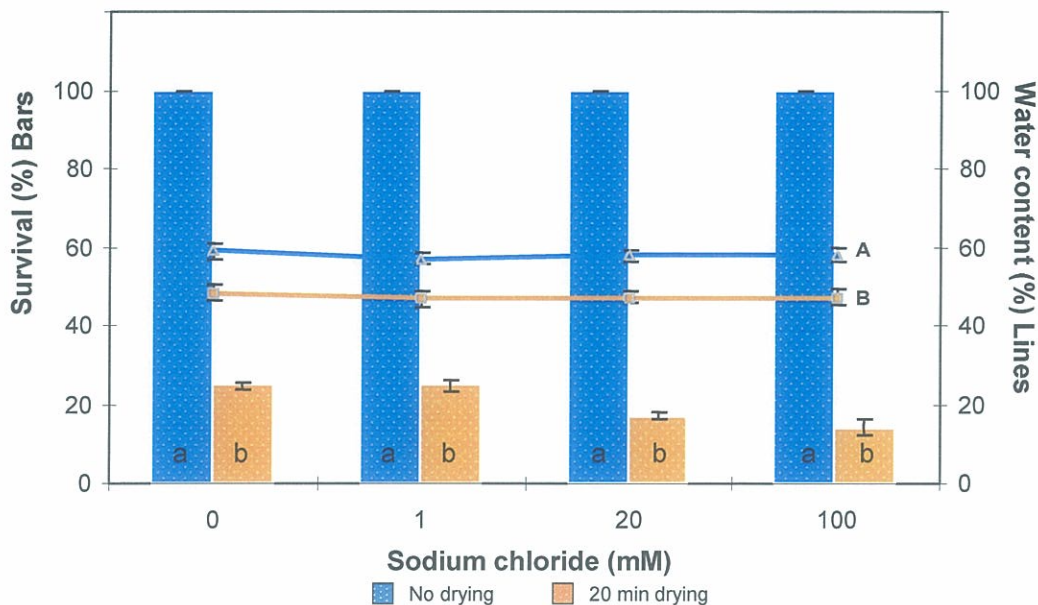


Figure 3.17: The drying response (lines) and survival (bars) of *E. grandis* axillary buds isolated from clusters that were cultured on ABA for 5 days, and thereafter on modified MS medium (40mM N- NO₃⁻), together with various concentrations of NaCl for 14 days. The mean water content is expressed as a percentage of the fresh weight, and the survival (expressed as percentage survival of the treated buds after 10 days on standard nutrient MS medium). Bars indicate standard errors and the different letters (a-b) indicate significant differences; n=20; p < 0.0001 (for survival) and p= 0.0003 (for water content).

The survival of the *E. grandis* axillary buds that were allowed to recover on the standard nutrient MS medium showed that after 20 minutes of drying, as the NaCl concentration increased, the survival of the *E. grandis* axillary buds decreased numerically but this was not significant (Figure 3.17). There was approximately a 75% loss of survival between the non dried and dried buds.

Gross morphology of *E. grandis* clusters cultured on standard MS nutrient medium together with ABA and NaCl for 14 days:

With increasing NaCl concentrations, the incidence of leaf tip browning, whole leaf browning and necrosis increased, whilst the number of clusters that did not demonstrate any injury decreased. The browning of the *E. grandis* clusters could be attributed to the presence of ABA

for longer than 5 days (in this case, 14 days), and, the addition of NaCl may have exacerbated or inflicted a secondary stress, thus causing a reduction in the number of clusters that demonstrated no injury. At 1mM NaCl, in the presence of ABA and standard MS nutrient medium, (Figures 3.18 and 3.19b), the incidence of leaf tip browning decreased, while whole leaf browning increased, thus indicating that ABA might have had some protective mechanisms that allowed a number of clusters to remain healthier. This trend was also observed in the treatment where *E. grandis* clusters were pre-treated on ABA for 5 days, and thereafter on nutrient MS medium with added NaCl for 14 days (Figure 3.12). However, as the NaCl concentration increased, the size of the clusters decreased. Statistical analysis indicated that as the NaCl concentration increased, the number of clusters that experienced no injury decreased (Figure 3.18).

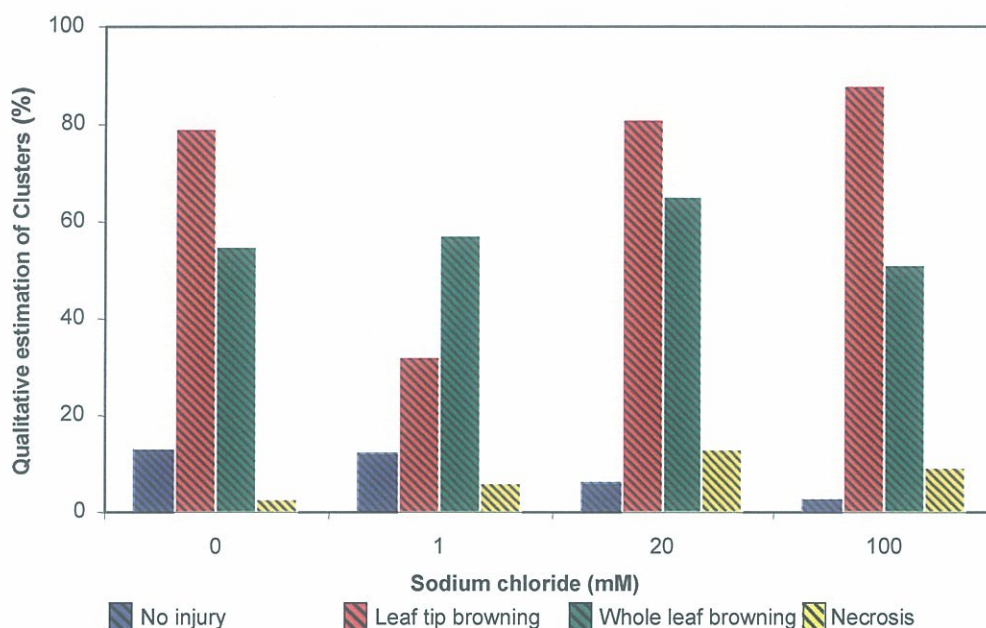


Figure 3.18: The degree of injury caused to *E. grandis* clusters that were cultured on unmodified nutrient MS medium, together with ABA and various concentrations of NaCl for 14 days. The degree of injury is expressed as a percentage of the total number of clusters cultured on the nutrient MS medium.

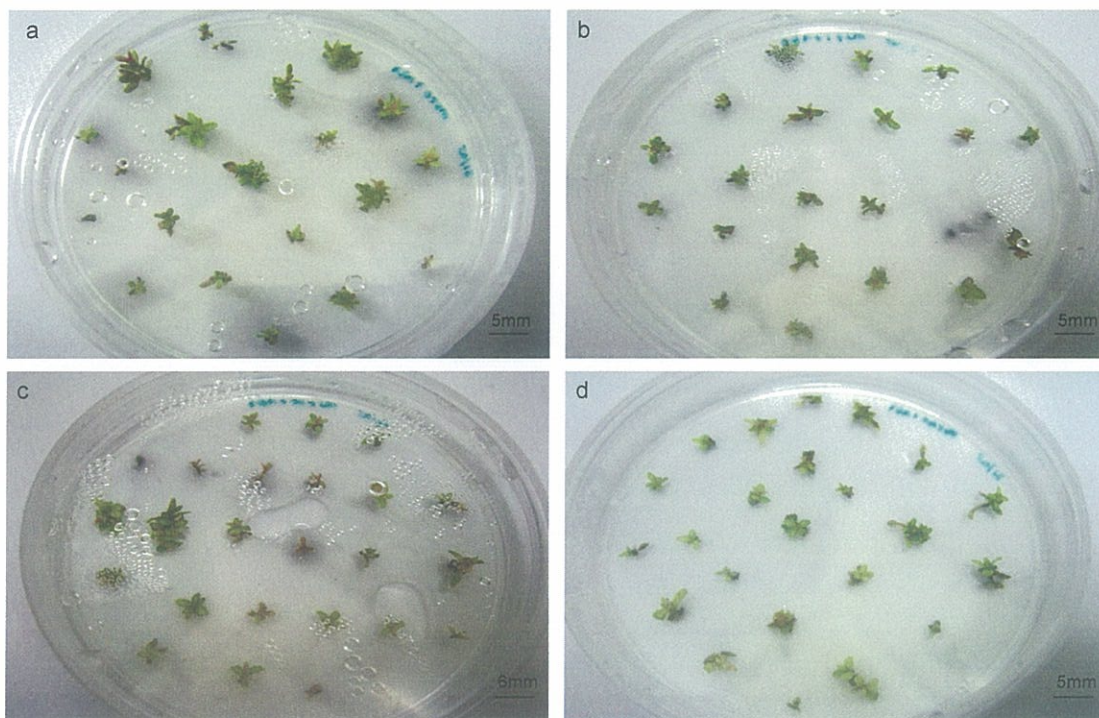


Figure 3.19: *E. grandis* clusters that were cultured on ABA and standard nutrient MS medium with added NaCl for 14 days (a) ABA + MS only (b) ABA + MS + 1mM NaCl (c) ABA + MS + 20mM NaCl (d) ABA + MS + 100mM NaCl.

The effect of desiccation on the *in vitro* *E. grandis* axillary buds, isolated from clusters that had been cultured on standard nutrient MS medium together with NaCl and ABA:

As previously observed (Figure 3.3), the presence of NaCl in the nutrient medium had no significant effect on the sample water content, both before and after 20 minutes of drying (Figure 3.20). The water content ranged from $59\% \pm 2.15$ (no drying) to $47\% \pm 1.95$ (after 20 minutes of drying), thus indicating that ABA did not improve the desiccation tolerance of the buds (Figure 3.20).

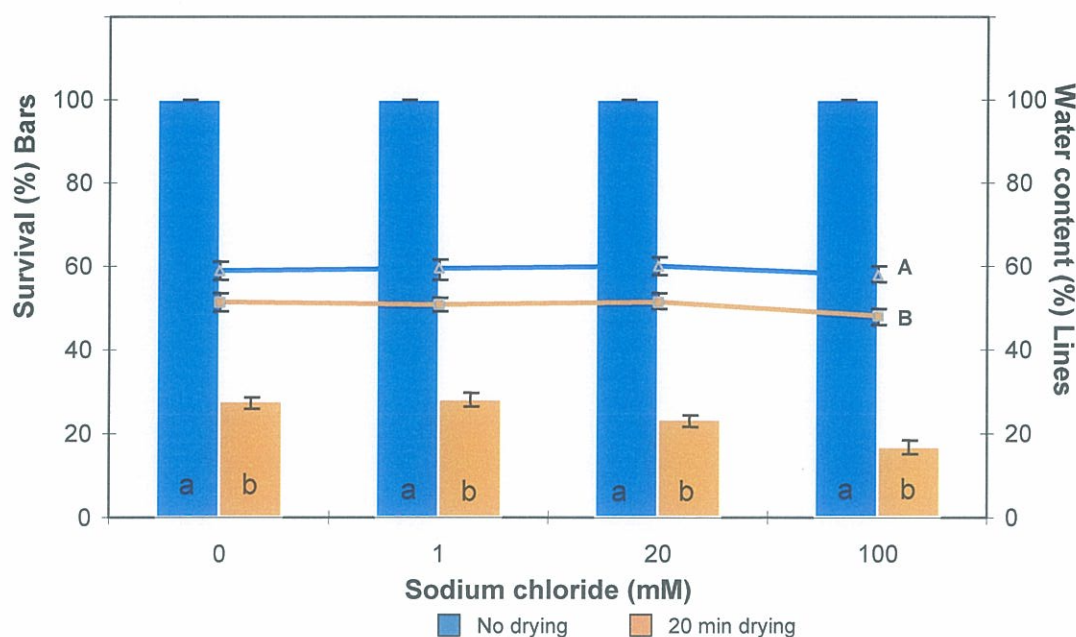


Figure 3.20: The drying response (lines) and the survival (bars) of *E. grandis* axillary buds isolated from clusters that have been cultured on standard MS medium, together with ABA various concentrations of NaCl for 14 days. The mean water content is expressed as a percentage of the fresh weight, and the survival (expressed as percentage survival of the treated buds after 10 days on standard nutrient MS medium). Bars indicate standard errors and the different letters (a-b) indicate significant differences; $n=20$; $p < 0.0001$ (for survival) and $p= 0.0026$ (for water content).

The *E. grandis* axillary buds showed an 80% loss in survival when they were desiccated for 20 minutes (Figure 3.20). When comparing this combination treatment with the treatment where the buds were first pre- treated with ABA for 5 days and thereafter standard nutrient MS medium with NaCl (Figure 3.12), the latter treatment showed a significant improvement (~ 7% increase) in the survival of the buds.

Gross morphology of *E. grandis* clusters cultured on modified MS nutrient medium (40mM N-NO₃⁻) together with the NaCl and ABA for 14 days: *Eucalyptus grandis* clusters that were cultured in the presence of a reduced nitrogen concentration (i.e. only nitrate on the modified MS nutrient medium), NaCl and ABA for 14 days all demonstrated some form of injury (Figure 3.21, Figure 3.22). Approximately 30% of the *E. grandis* clusters did not survive the treatment.

Statistical analysis indicated that clusters that had no signs of injury and necrosis showed no association between the type of injury and the NaCl concentrations, however, leaf tip browning, and whole leaf browning demonstrated a negative interaction between the type of injury and the NaCl concentration. As the NaCl concentration increased, the number of clusters that expressed injury increased (Figure 3.21). Furthermore, as the concentration of NaCl increased, the size of the clusters decreased, and the shoots on the clusters were more tightly packaged (Figure 3.22).

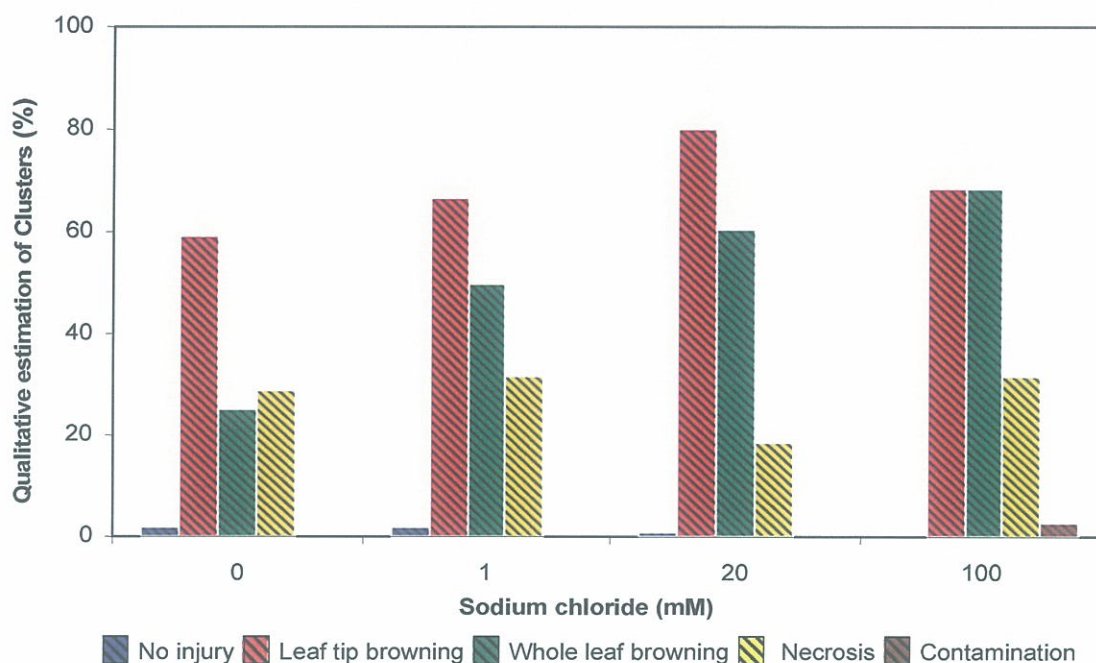


Figure 3.21: The degree of injury caused to *E. grandis* clusters that were cultured on modified nutrient MS medium (40mM NO_3^-) together with ABA and various concentrations of NaCl for 14 days. The degree of injury is expressed as a percentage of the total number of clusters cultured on the modified nutrient MS medium.

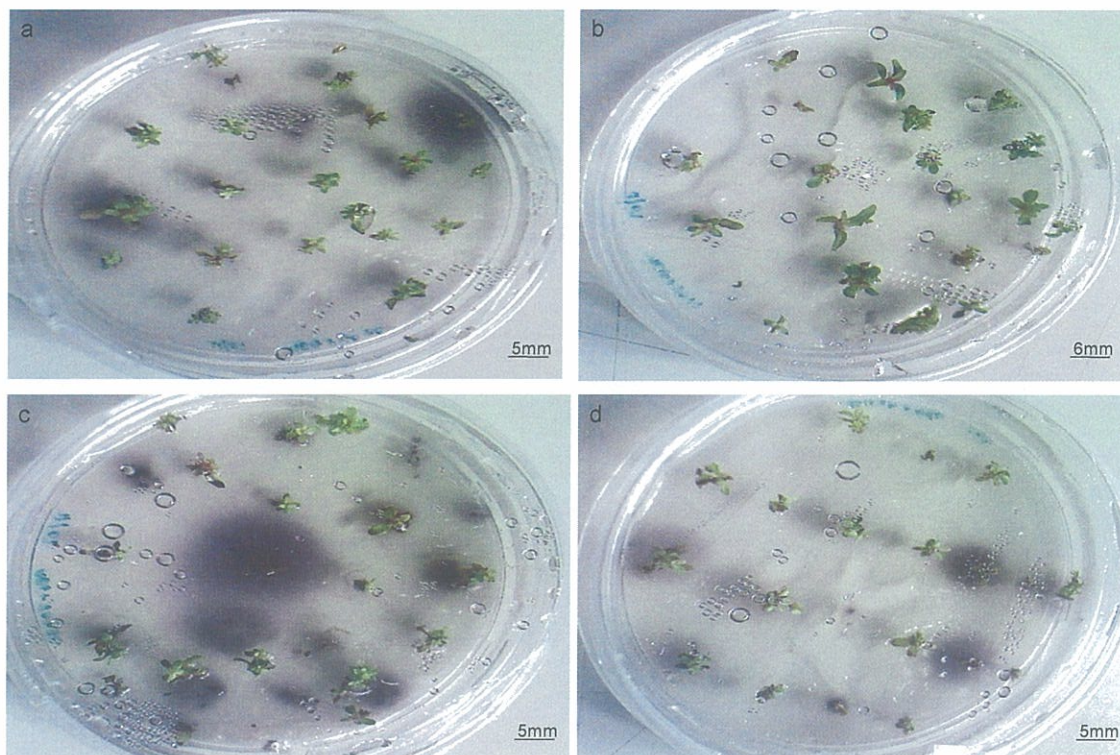


Figure 3.22: *E. grandis* clusters that were cultured on ABA and modified nutrient MS medium (40mM NO_3^-) with NaCl for 14 days (a) ABA + 40mM NO_3^- only (b) ABA + 40mM NO_3^- + 1mM NaCl (c) ABA + 40mM NO_3^- + 20mM NaCl (d) ABA + 40mM NO_3^- + 100mM NaCl. Note in all case, tannins/ polyphenolics were produced.

The effect of desiccation on the *in vitro* *E. grandis* axillary buds, isolated from clusters that had been cultured on modified nutrient MS medium (40mM N- NO_3^-) together with NaCl, and ABA for 14 days: The water content of *E. grandis* axillary buds that were cultured on the modified nutrient MS medium (40mM N- NO_3^-), NaCl, and ABA for 14 days showed no significant difference between the NaCl concentrations and the drying times (Figure 3.23). The water content ranged between $58\% \pm 1.94$ (after no drying) and $53\% \pm 2.11$ (after 20 minutes of drying) (Figure 3.23). This implied that the removal of ammonium and the addition of ABA to the nutrient medium had a positive effect on the plants ability to lose water. The survival of buds that were cultured on the modified nutrient medium showed an 80% loss of viability when the

buds were desiccated for 20 minutes (Figure 3.23). Again, this could be attributed to the double stress inflicted on *E. grandis* axillary buds (the addition of ABA and NaCl to the medium for 14 days).

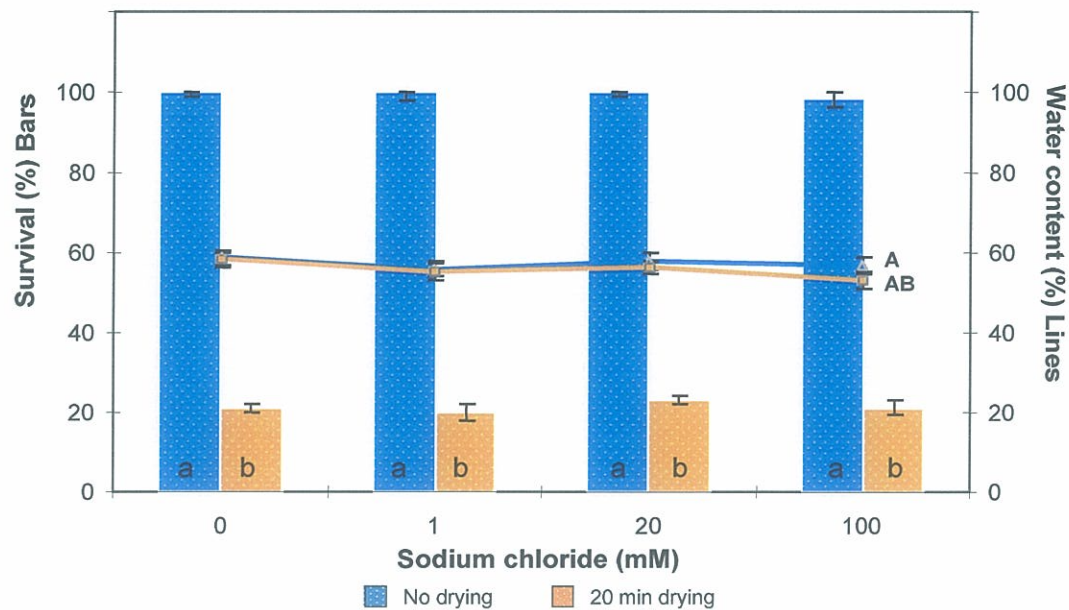


Figure 3.23: The drying response (lines) and the survival (bars) of *E. grandis* axillary buds isolated from clusters that were cultured on modified MS medium (40mM NO_3^-), together with ABA and various concentrations of NaCl for 14 days. The mean water content is expressed as a percentage of the fresh weight, and the survival (expressed as percentage survival of the treated buds after 10 days on standard nutrient MS medium). Bars indicate standard errors and the different letters (a-b) indicate significant differences; $n=20$; $p < 0.0001$ (for survival) and $p > 0.05$ (for water content).

4. DISCUSSION

4.1 Effect of NaCl and Nitrogen on *E. grandis* growth.

The composition of the plant growth medium, as well as the exposure of plants to periods of drought will reduce the growth of the plant. Plants that are grown under saline conditions most often experience reduced growth and suffer from water deficit (Hu and Schmidhalter, 2005). Under water deficit conditions, the plant cells tend to reduce the uptake and transportation of important nutrients essential for plant growth (Grattan and Grieve, 1999). This could possibly be protecting the plant from the uptake of toxic ions (Kozlowski and Pallardy, 2002; Qadir, *et al.*, 2008), especially if the concentration of these ions is high in the growth medium. In some plant species, the addition of NaCl to the plant growth nutrient medium, can affect the water content within the cells of plants. In this regard, Morabito *et al.*, (1994) observed that the *Eucalyptus microtheca* that was exposed to NaCl *in vitro*, displayed an increase in the Na⁺ and Cl⁻ accumulation, and an increase in the water content of the plant cells (Morabito *et al.*, 1994). Another possibility is that the plant cells may accumulate Na⁺ and Cl⁻ ions, an interaction that may result in nutrient imbalances, deficiencies, and toxicity (Sairam and Tyagi, 2004, Inal, *et al.*, 2009).

The addition of NaCl to the nutrient medium in the present 14 day experiments caused the *E. grandis* explants to grow more slowly and to exhibit observable growth injuries with increasing NaCl concentrations (Figure 3.2). In some experiments, there was evidence of the leaves becoming lighter (Figure 3.22) or even red (Figure 3.16). This could possibly be attributed to the NaCl (since chlorine is a bleaching agent). This in turn could reduce the photosynthetic abilities of the *E. grandis* cluster which could have lead to the noticeable leaf lightening, reddening as the underlying red pigments could be exposed, and then leaf browning as the remaining photosynthetic pigments were bleached. A similar observation was made by Morabito, *et al.*, (1996) where *in vitro* grown *Eucalyptus microtheca* showed a reduced growth rate with increasing NaCl concentrations. Ions are known to accumulate in the plant vacuoles (Hasegawa, *et al.*, 2000; Zhu, 2001; Yokoi *et al.*, 2002) hence, further investigations should be conducted to determine if Na⁺ and/ or Cl⁻ ions are accumulating within vacuoles of the *E. grandis* axillary

buds. This would provide insight in the level of ion toxicity that the *E. grandis* tissue may be experiencing.

Nitrogen is an element that plants require in large quantities as it is a constituent of many plant cell components, such as amino and nucleic acids. Higher plants are capable of using nitrate and ammonium as nitrogen sources. It is for this reason that both nitrate and ammonium are supplied in the MS nutrient medium. Thus, a deficiency in nitrogen rapidly inhibits plant growth (Hu, and Schmidhalter, 2005).

It is well known that ammonium accumulation can produce toxic effects (Britto and Kronzucker, 2002) and reduced growth rates whereas in contrast most plants can tolerate excess concentrations of nitrate and its accumulation within their tissues (Lasa, *et al.*, 2001). Consequently, it was decided to eliminate NH_4^+ (which is taken up passively by the plant) from the nutrient medium as an attempt for the plant to take up nitrate and to gain lower water content and a high survival rate post desiccation. Results obtained from the present investigation demonstrated that the removal of N- NO_3^- from the culture medium lead to the death of the *E. grandis* axillary buds (Figure 3.8), thus further supporting the statement that N- NH_4^+ accumulation is toxic to plants. Sensitivity of plants to ammonium nutrition is often expressed as growth suppression, which in turn could lead to the necrosis of the cultured plants (Britto and Kronzucker, 2002).

Chloride ions are known to interact with the level of nitrogen uptake i.e. an increase in Cl^- will decrease the uptake of NO_3^- (Grattan and Grieve, 1999; Kozłowski, 1997). As the concentration of NaCl increases, the uptake of Cl^- will increase, therefore leading to a decrease in the uptake of NO_3^- . *E. grandis* axillary buds exposed to NaCl (in particular the higher NaCl concentrations) on the modified nutrient medium (only N- NO_3^-) demonstrated an increased level of injury and this lead to a lower survival rate post desiccation (Figure 3.6). In contrast, *E. grandis* explants that were cultured on the full concentration of nitrogen (20mM N- NH_4^+ and 40mM N- NO_3^-) in the presence of NaCl displayed a higher survival rate (Figure 3.2). From the results obtained, it is possible that chloride ions were taken up instead of nitrate ions, demonstrated by the bleaching of leaves (Figure 3.5). It is also possible the slow growth rate of plants under saline conditions

prevented the dilution effect of accumulated ions in the plant vacuole (due to the plant taking up more water so as to dilute the accumulated ion), thus, higher water content and a lower survival rate of *E. grandis* axillary were recorded.

4.2 Effect of desiccation on *E. grandis* axillary buds

Tolerance to desiccation can be classified as the ability of the cells to survive the dehydration process and resume normal cell functioning upon rehydration. One of the most crucial requirements for successful cryopreservation is reducing the water content within the selected plant organ/cell/ tissues in order to reduce the formation of intracellular ice crystals during the freezing and thawing processes. Since the critical water content within each plant organ/ tissue/ cell varies, different methods and rates of desiccation may have influenced the ability of the *E. grandis* axillary buds to tolerate desiccation (Wesley- Smith, *et al.*, 2001). For this reason several investigations in our laboratory have considered different methods to achieve different rates of drying. Drying in the laminar flow allowed for material to be dried to water contents suitable for cryostorage but there was a considerable variation in the water content of the material. Mlambo (2004) attempted to equilibrate axillary buds to specific RHs and thus set water contents. However, whilst this approach reduced water content variability, the rate of drying was too slow and the material died. Padayachee *et al.*, (2008) showed that desiccation over activated silica gel in a set volume, reduced water content both sufficiently fast and without variability. This approach was therefore adopted in the present experiments.

Desiccation damage can arise when the species tolerance mechanisms fail to maintain the water balance/ homeostasis necessary for survival (Walters, *et al.*, 2002) as an effect of the desiccation / drying process being too extreme. Bud excision prior to desiccation may have exacerbated the injury caused to the buds during drying but, as the material did not lose viability, this was considered to be minimal. However, the removal of water from the plant cells (in preparation for cryopreservation) also alters the physical and physiological properties of the cells (Walters, *et al.*, 2002) and damage may be purely based on the removal of water from the cell, and not the processes prior to removal (such as excision). The water content of plant tissues varies amongst species and the influence of plant growth medium may also be influential for *in vitro* cultured

plants. The exposure to NaCl and desiccation stress triggers cellular dehydration in plants and this in turn causes osmotic stresses and removal of water from the cytoplasm into the extracellular spaces. The formation of reactive oxygen species (ROS) is another effect that results from drought and salt stress. In an attempt to reduce the water content of the *E. grandis* axillary buds by means of NaCl pretreatment followed by desiccating the buds, the plant tissue may have produced and accumulated ROS. This may have had a negative impact on the cellular structures and in turn on the metabolism of the plant cell (Bartels and Ramanjulu, 2005). This in turn may have caused the observed increase in browning of the *E. grandis* tissues (Figures 3.5, 3.7, 3.10(b), 3.13, 3.16, 3.19 and, 3.22). Thus, the continuous stresses applied to the plant cells (i.e. NaCl, excision, and desiccation) possibly enhanced the generation of ROS, and since the generation of ROS may result in the apoptosis of plant cells (Bartels and Ramanjulu, 2005). This could account for the low survival rate of plant cells.

The effect of desiccation on the *E. grandis* axillary buds cultured on standard Murashige and Skoog (1962) with added NaCl was investigated to determine if the addition on NaCl would assist in reducing the water content of the buds in preparation for cryopreservation. The results obtained indicated that the addition of NaCl in combination with different drying periods did not assist in lowering the water content of the buds i.e. they dried to the same water content in the same drying period. It is possible that the addition of NaCl to the nutrient medium could have caused the plant to take up Na^+ and Cl^- , and thereafter compartmentalize it, most probably in the vacuoles. As the subcellular Na^+ and Cl^- concentrations increased the cells/ tissues would have taken up more water from the nutrient medium in attempt to dilute the ions. This was measured as increased water content of the axillary buds and reduced amounts of water being lost during the 20 minutes of drying (Figure 3.3). Future experimental work examining the uptake of Na^+ and Cl^- ions could clarify the likelihood of this being true.

Bewley (1997) and Bohnert and Cushman (2000) suggest that there are two categories into which desiccation tolerant plants may fall: (a) those that withstand desiccation by relying on protective mechanisms within the cells and (b) those that can withstand desiccation by repairing damage caused through the desiccation process. Both of these processes make use of the sugars and proteins within the cells. The uptake of sugars and generation of new protein (e.g. LEA's -

see earlier) proteins assist the plant cells in preventing damage and / or repair cells by taking up additional water to compensate for the water loss during desiccation. Water has a structural role in plant cells. At the plant cell level, water fills in the vacuole, thus providing turgor support. The minimal water loss experienced by *E. grandis* buds (thus the high water content) (Figures 3.3, 3.6, 3.8, 3.11, 3.14, 3.17, 3.20, and 3.23) could be a result of increased water taken up to repair damaged cells with the aid of sugars and proteins. At the molecular level, water presents the hydrophilic and hydrophobic interactions that are necessary for the protein and lipid conformation within the cytomatrix and organelles (Walters, *et al.*, 2002), thus providing additional mechanisms for repairing desiccation damaged cells. The minimal water loss and the low survival rate demonstrated by *E. grandis* that were cultured on the different nutrient medium (i.e. standard nutrient MS medium with the full nitrogen content and the modified nutrient medium with 40mM NO₃⁻ - Figures 3.3 and 3.6), indicated that cellular and molecular interferences were experienced during the treatment of *E. grandis* clusters and desiccation of buds thus making the buds unsuitable for cryopreservation. If the uptake of additional water was the cause for minimal water loss, then the survival post desiccation should have been higher. However, this was not seen when axillary buds were allowed to recover following desiccation, and thus the uptake of the deleterious Na⁺ and Cl⁻ ions seems more probable. As a consequence, the addition of NaCl to cross trigger desiccation tolerance can be discarded as the NaCl essentially promoted the death of the axillary buds post desiccation. Thus, the use of NaCl to assist in reducing the water content of the buds for cryopreservation is not a viable procedure for these tissues.

4.3 Effect of ABA on stressed *E. grandis* axillary buds

Absciscic acid is a regulator that coordinates responses to environmental stresses. This may be beneficial to the plant as the ABA induces changes at the cellular, tissue, organ, and whole plant level (Xiong and Zhu, 2003). Drought stress imposed on plants triggers the production of ABA, which initially causes stomatal closure to minimize water loss in plants. ABA also expresses stress responsive genes that encode enzymes for the biosynthesis of compatible osmolytes and LEA-like proteins, which collectively increase plant cell, tissue and organ stress tolerance (Hasegawa, *et al.*, 2000).

The exogenous application of ABA may lead to the induction of stress response pathways that may induce drought tolerance genes (Xiong and Zhu, 2003). It was for this reason ABA was included in the nutrient media used to culture the *E. grandis* material. In plant tissue culture, salinity may affect the growth of *in vitro* plants by minimising the cell water potential through the accumulation of ions or incompatible solutes (Mills, *et al.*, 2001). The uptake of the salt ions may be toxic to the plant as they have the ability to inhibit the uptake of other minerals (see Chapter 1: 1.3.2- Salt Stress). Mills, *et al.*, (2001) showed that the exogenous application of ABA to *in vitro* grown Jojoba plants had a similar effect to those that were grown only on the saline media and also noted that various ions such as Na^+ , K^+ , Ca^{2+} , and Cl^- accumulated in Jojoba shoots. This could account for the minimal water loss experienced by *E. grandis* axillary buds - it is possible that the plant cells were taking up- and accumulating Na^+ and Cl^- (as these ions compete with K^+ and NO_3^- respectively). Further investigations need to be conducted to test for the accumulation of Na^+ , K^+ and Cl^- ions in the cytoplasm and vacuole of *E. grandis* shoots and to determine if they influenced the dry weight of the buds.

In several of the performed experiments, ABA was supplied to the *E. grandis* shoots via the nutrient medium containing NaCl (1, 20 and 100mM NaCl) and either the full concentration of nitrogen (20mM NH_4^+ + 40mM NO_3^-) or the modified form (only 40mM NO_3^-). The combined effect of NaCl and ABA, and the initial pre-treatment with ABA was examined to determine which method produced axillary buds with lower water contents after drying yet maintained viability. The ABA concentration of 5mg/l (Padayachee, 2000) in combination with NaCl or as a 5 day pre-treatment, was sufficient to inhibit shoot elongation and water loss especially at higher NaCl concentrations (Figure 3.13c-d and 3.19). Similar observations were noted when ABA was supplied in the nutrient medium with only 40mM NO_3^- and NaCl (Figure 3.16 and 3.22) thus indicating that ABA did not have a positive effect in reducing the water content of the buds in preparation for cryopreservation.

The production of phenolics in culture is characteristic of wounding and stress amongst plants. This could indicate the initiation of stress response pathways that are being triggered within the plant tissues. Phenolics are usually produced in response to the oxidative burst (ROS) reported to

occur as part of the stress signalling pathways (Padayachee *et al.*, 2008). Padayachee, *et al.*, 2008 also noted the production of phenolics in response to the addition of ABA, therefore indicating that phenolic production was normal when *E. grandis* was cultured on 5mg/ml ABA for 5 days. However, in the present experiments, the addition of NaCl to the nutrient medium, in addition to ABA, may have exacerbated the extent of phenolic production. It is possible that phenolics were produced (Figure 3.22) as a response feedback mechanism to ameliorate the ROS that were produced as a result of ABA and/ or NaCl stress (Purohit, *et al.*, 1992). Thus, *E. grandis* demonstrated a protective mechanism which could have further accounted for the reduced water loss in the buds (Figures 3.11, 3.14, 3.17, 3.20, 3.23) (Padayachee, *et al.*, 2008).

Under conditions of abiotic stress, the concentration of endogenous ABA increases in response to stress within the cells (Skriver and Mundy, 1990). A response to water deficit at both a cellular and molecular level results in the accumulation of osmolytes and proteins that are specifically involved in stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 2007). The plants' ability to adjust to the addition of NaCl and desiccation could also trigger the increase in ABA levels in plant cells (Bohnert, *et al.*, 1995). It is possible that both the naturally produced ABA levels and the externally supplied ABA (present in the nutrient medium) were adequate for minimizing the number of plants experiencing necrosis at high NaCl concentrations (Figure 3.12). This in turn could be important for the desiccation tolerance of *E. grandis* explants. In the presence of only nitrate as the nitrogen source (Figure 3.15), the 5 day ABA pre-culture was not important for the survival of *E. grandis* clusters as compared with those clusters that were cultured on the full concentration of nitrogen (both NH_4^+ and NO_3^-) in the standard MS nutrient medium. This could indicate that the removal of NH_4^+ from the nutrient medium had an effect on the ability of the *E. grandis* buds to survive (Figure 3.17). Thus in the absence of NH_4^+ , ABA did not improve the ability of the buds to survive desiccation; however, this would be expected since the buds which were nutrient deficient, were presented with a double stress (i.e. ABA and NaCl for 14 days). Even though the buds retained their water, it appeared that all other survival mechanisms had been turned off (or were not even present) and thus the viability of the material was compromised (Figures 3.11, 3.14, 3.17, 3.20, 3.23).

4.4 Cross stress

Drought, salinity and temperature changes cause major limitations on plant growth. These stresses are complex environmental phenomena that involve the simultaneous operation of many signaling pathways (Gasper *et al.*, 2002). To determine if cross tolerance was occurring in the present set of experiments, genes that are common (or have a similar response) in the salt stress, ABA and drought pathways could have been examined. This would have determined if the signaling pathways shared common components during times of stress. Thus, only speculation on these can be reported upon. The involvement of common genes and their triggers would have provided a deeper understanding of the stress pathways and physiological and cellular changes that are a result thereof e.g. plasma membrane changes, cellular turgidity, cellular damage etc. (Bohnert, *et al.*, 1995). However, such molecular approaches are expensive and time consuming. The aim of this research was to determine whether there were any indications of cross talk/ tolerance under these experimental conditions. Subsequent in depth investigations could have been initiated if positive indicators had been obtained.

Gazzarrini and McCourt (2003) considered the interaction of ethylene, ABA and GA, and reported that the hormones appear to interact at different levels where they share signaling components that create nodes of interactions. The common pathways required for the biosynthesis of plant hormones indicated how the phenomenon of cross talk can cause different responses in different organs. The supplementation of ABA and NaCl to the nutrient MS medium was conducted in anticipation that the water content of the axillary buds would decrease and thus the *E. grandis* axillary buds would be at optimal water content for cryopreservation. However, this did not occur in *E. grandis* material, and the cascade of deleterious reactions as seen in *E. grandis* may suggest that the mechanism of ‘cross-talk’ between the NaCl and /or ABA pathways did not exist. Nevertheless, results from the presented experiments could be used to enhance future research and examine the role of different concentrations of hormones (other than ABA) and their possible role in assisting to lower the water content of the *E. grandis* axillary buds. The additional information will provide insight on whether hormonal signaling in combination with low concentrations of NaCl is evident, and how to further prepare the plant tissue in order to achieve successful cryopreservation.

4.5. Future Work

A potential method of increasing the salt tolerance and thus achieving lower water content (in preparation for cryopreservation) of *E. grandis* would be to grow the plants in the greenhouse while watering them with saline solutions. There are two ways this could be done to determine the tolerance of *E. grandis* to NaCl:

- Water a selected number of plantlets with NaCl solutions and weekly increase the concentration of NaCl.
- Water a selected number of plants with one concentration of NaCl only. For example, water 10 plants with 1mM NaCl, 10 plant with 10mM NaCl, 10 plants with 50mM NaCl, and 10 plant with 100mM NaCl.

Monitor the physiological changes (such as changes in colour of leaves, new shoot formation, rate at which the plant grows) that occur and measure the weight (fresh and dry weight) of a few of the buds from the plants. During each stage, a few buds should be sectioned and examined with the light microscope to identify if there is a change in the physiology of plant cells. *E. grandis* explants can then be established *in vitro* (on nutrient medium containing NaCl), and the accumulation of ions can be measure by assaying the culture medium after the plants have been cultured the media for the desired timeframe. The results should correlate to those obtained from the water content. If the results from this experiment are fruitful (i.e. low water content is achieved with a high survival rate), then *E. grandis* material can be successfully grown *in vitro* and thereafter, tested for their amenability to cryopreservation.

Future studies detailing the biochemical and molecular mechanisms that plants use to adapt to environmental stresses is another necessary step for better understanding of approaches to improve plant growth under stress. This will in turn lead to an enhanced understanding of plant metabolic pathways and the limits placed on their survival.

5. CONCLUSION

The improvement of *E. grandis* explants' tolerance to desiccation by exposure to salt stress was not successful. It is possible that the stress introduced to the *E. grandis* clusters lead to a disturbance of ion homeostasis, thus causing the ions to compete for uptake and entry into the cells. As a consequence, a minimal water loss and low survival rate was recorded, and thus the successful cryopreservation of *E. grandis* axillary buds would not be feasible using the reported approach.

6. REFERENCES

- Alpert, P. (2000). The discovery, scope and puzzle of desiccation tolerance in plants. *Plant Ecology* 151, 5-17.
- Anonymous. (1993). South African Forestry. Promotion Committee, Forestry Council, Rivonia, South Africa.
- Anonymous. (2008). Forestry South Africa. Abstract of South African Forestry Facts for the year 2006/2007. Source Department of Water Affairs and Forestry. www.forestry.co.za
- Arbothnot, A. (2001). Clonal testing of *Eucalyptus* at Mondi Kraft, Richards Bay. *Forestry Genetics for the Next Millennium*, pp61-65. Proceedings of The International Union of Forest Research Organization (IUFRO) Working Party, Durban, South Africa.
- Argita, L., Fernandez, A. G., Gonzalez, A., and Tames, R. S. (2005). Effects of the application of benzyladenine pulse on organogenesis, acclimatisation and endogenous phytohormone content in kiwi explants cultured under autotrophic conditions. *Plant Physiology and Biochemistry* 432, 161-167.
- Baek, H., Kim, H., Cho, E., Chae, Y., and Engelmann, F. (2003). Importance of explant size and origin and of preconditioning treatments for cryopreservation of garlic shoot apices by vitrification. *CryoLetters* 24, 381-388.
- Bartels, D., and Salamini, F. (2001). Desiccation tolerance in the resurrection plant *Craterostigma platagineum*: A contribution to the study of drought tolerance at the molecular level. *Plant Physiology* 127, 1346-1353.
- Bartels, D. and Ramanjulu, S. (2005). Drought and salt tolerance in plants. *Critical Reviews in Plant Sciences* 24, 23-58.
- Benson, E. E. (1994). Cryopreservation (pp 235-267) in Dixon, R. A. and Gonzales, R. A (Eds). *Plant Cell Culture: A Practical Approach* (second edition). IRL Press, Oxford.
- Benson, E. E. (2008). Cryopreservation of Phytodiversity: A critical appraisal of theory and practice. *Critical Review in Plant Sciences* 27, 141-219.
- Bewley, J. D. (1997). Seed germination and dormancy. *The Plant Cell* 9, 1055-1066
- Biddington, N. L. (1992). The influence of ethylene in plant tissue culture. *Plant Growth Regulations* 11, 173-187.
- Blake, T. J. (1981). Salt tolerance of Eucalypt species grown in saline culture. *Australian Forestry Research* 11, 179-183.

Bohnert, H. J., Nelson, D. E., and Jensen, R. G. (1995). Adaptations to environmental stress. *The Plant Cell* 7, 1099-1111.

Bohnert, H. J., and Cushman, J. C. (2000). The ice plant cometh lesson in abiotic stress tolerance. *Journal of Plant Growth Regulation* 19, 334-346.

Bowler, C., and Fluhr, R. (2000). The role of calcium and activated oxygen as signals for controlling cross tolerance. *Trends in Plant Science*, 5 241-246.

Britto, D. T and Kronzucker, H.J. (2002). NH_4^+ toxicity in higher plants: a critical review. *Journal of Plant Physiology* 159, 567-584.

Das, T., and Mitra, G. C. (1990). Micropropagation of *Eucalyptus tereticornis* Sm. *Plant Cell Tissue and Organ Culture* 22, 95-103.

Davies, P. J. (1995). The Plant Hormones: Their Nature, Occurance and Function. In Davies, P. J. (Ed). *Plant Hormones: Physiology, Biochemistry and Molecular Biology*. Kluwer Academic Press. The Netherlands.

Dumet, D., Engelmann, F., Chabrillange, N., Duval, Y., and Derueddre, J. (1993). Importance of sucrose for the acquisition of tolerance to desiccation and cryopreservation of oil palm somatic embryos. *CryoLetters* 14, 243-250.

Durand- Cresswell R. J., Boulay, M., and Franclet, A. (1982). Vegetative propagation of *Eucalyptus*, pp150-181. In Bonga, J. M., and Durzan, D. J (Eds). *Tissue Culture in Forestry*. Martinus hijhoff, The Hague.

Finkelstein, R. R., Gampala, S. S. L., Rock, C. D. (2002). Absciscic acid signaling in seeds and seedlings. *The Plant Cell (Suppl.)*, S15-S45.

Fuller, B. J. (2004). Cryoprotectants: the essential antifreezes to protect life in the frozen state. *CryoLetters* 25, 375-388.

Gaspar, T., Franck, T., Bisbis, T., Kevers, C., Jouve, L., Hausman, J. F., and Dommes, J. (2002) Concepts in plant stress physiology. Application to plant tissue culture. *Plant Growth Regulation* 37, 263-285.

Gazzarrini, S., and McCourt, P. (2003). Cross- talk in plant hormone signaling: what Arabidopsis mutants are telling us? *Annals of Botany* 91, 605-612.

George, E. (1996). *Plant propagation by tissue culture (part 2), in practice*. Exergetics Limited, Edington.

Grattan, S. R., and Grieve, C. M. (1999). Salinity- mineral nutrition in horticultural crops. *Scientia Horticulture* 78, 127-157.

- Hammet, N. (1992). Progress in the biotechnology of trees. *World Journal of Microbiology and Biotechnology* 6, 369-377.
- Hasegawa, P. M., Bressan, R. A., Zhu, J. K., and Bohnert, H. J. (2000). Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology & Plant Molecular Biology* 51, 463-499.
- Helliot, B., Swennen, R., Poumay, Y., Frison, E., Lepoivre, P., and Panis, B. (2003). Ultrastructure changes associated with cryopreservation of banana (*Musa spp.*) highly proliferating meristems. *Plant Cell Reports* 21, 690-698.
- Himmelbach, A., Yang, Y., and Grill, E. (2003). Relay and control of abscisic acid signaling. *Current Opinion in Plant Biology* 6, 470-479.
- Hoekstra, F.A., Golovina, E.A., and Buitink, J. (2001). Mechanisms of plant desiccation tolerance. *Trends in Plant Science* 6, 431-438.
- Hu, Y., and Schmidhalter, U. (2005). Drought and salinity: A comparison of their effects on mineral nutrition of plants. *Journal of Plant Nutrition and Soil Science* 168, 541-549.
- Inal, A., Gunes, A., Pilbeam, D. J., Kadioglu, Y. K., Eraslan, F. (2009). Concentrations of essential and nonessential elements in shoots and storage roots of carrot grown in NaCl and Na₂SO₄ salinity. *X-Ray Spectrometry* 38 45-51.
- Jakab, G., Ton, J., Flors, V., Zimmerli, L., Metraux, J. P., and Mani, B. M. (2005). Enhancing *Arabidopsis* salt and drought stress tolerance by chemical priming for its Absciscic acid response. *Plant Physiology* 139, 267- 274.
- Joshi, I., Bisht, P., Sharma, V. K., and Uniyal, D. P. (2003). *In vitro* clonal propagation of mature *Eucalyptus* F₁ hybrid (*Eucalyptus tereticorrus* Sm. X *E. grandis* Hill Ex. Maiden). *Salvae Genetica* 52, 110-113.
- Kadlecova, Z., Faltus, M., and Prasil, I. (2000). The relationship between abscisic acid content, dry weight, and freezing tolerance in Barley CV. Lunet. *Journal of Plant Physiology* 157, 291-297.
- Kartha, K. K. (1985). Meristem culture and germplasm conservation, pp115-134. In Kartha, K. K. (Ed) *Cryopreservation of Plant Cells and Organs*. CRC Press, Boca Raton Florida.
- Kende, H., and Zeevaart, J.A.D. (1997). The five 'Classical' plant hormones. *The Plant Cell* 9, 1197- 1210.
- Klobus, G., Ward, R., and Huffaker, R. C. (1988). Characteristics of injury and recovery of net NO₃⁻ transport of Barley seedlings from treatments of NaCl. *Plant Physiology* 87, 878-882.

- Knight, H., and Knight, M. R. (2001). Abiotic stress signaling pathways: specificity and cross talk. *Trends in Plant Science* 6, 262-267.
- Konishi, M., and Yanagisawa, S. (2005). Signaling crosstalk between ethylene and other molecules. *Plant Biotechnology* 22, 401-407.
- Kozlowski, T. T. (1997). Response of woody plants to flooding and salinity. *Tree Physiology Monograph* 1, 1-17.
- Kozlowski, T. T., and Pallardy, S. G. (2002). Acclimation and adaptive responses of woody plants to environmental stresses. *The Botanical Review* 68, 270-334.
- Krogstrup, P., Baldursson, S., and Norg, A. (1992). *Ex situ* conservation by tissue culture. *Opera Botanica* 113, 49-53.
- Lakshmi Sita, G. (1986). Progress towards the *in vitro* clonal propagation of *Eucalyptus grandis*, pp159-165. In: Withers, L. A., and Anderson, P. G. (Eds) *Plant tissue culture and its agricultural applications*. Butterworths, London.
- Lambardi, M., Benelli, C., De Carlo, A. (2005). Cryopreservation as a tool for the long- term cryopreservation of woody plant germplasm development of technology at the CNR/VALSA Institute of Florence. *The Role of Biotechnology*. Villa Gualino, Turin, Italy.
- Lasa, B., Frechilla, S., Lamsfus, C., Apricio- Tejo, P. M. (2001). The sensitivity to ammonium nutrition is related to nitrogen accumulation. *Scientia Horticulture* 91, 143-152.
- Louw, W. J. A. (2004). General history of South African forestry industry: 1975- 1990. *South African Forestry Journal* 200, 77-86.
- Loyola- Vargas, W. M., De- La- Pena, C., Galaz- Avalos, R. M., Quiroz-Figueroa, F. R. (2008). *Plant Tissue Culture*, pp875. In Walker, J.M., and Rapley, R. (Eds). *Molecular Biomenhods Handbook, Second Edition*. Humana Press, Totowa, NJ.
- Ma, S., Gong, Q., Bohnert, H.J. (2006). Dissecting salt stress pathways. *Journal of Experimental Botany* 57, 1097- 1107.
- Mabvurira, D., and Pukkala, T. (2002). Optimizing the management of *Eucalyptus grandis* (Hill) Maiden plantations in Zimbabwe. *Forestry Ecology Management* 166, 149-157.
- Mansour, M. M. F., and Salama, K. H. A. (2004). Cellular basis of salinity tolerance in plants. *Environmental and Experimental Botany* 52, 113-122.
- McComb, J. A., and Bennett, I. J. (1986). *Eucalyptus*, pp340- 362. In: Bajaj, Y. P. S. (Ed). *Biotechnology in agriculture and forestry 1*. Springer- Verlag, Berlin.

Mehra – Palta, A. (1982). Clonal Propagation of *Eucalyptus* by Tissue Culture. *Plant Science Letters* 26, 1-11.

Meryman, H. T., and Williams, R. J. (1985). Basic principles of freezing injury to plant cells: natural tolerance and approaches to cryopreservation pp13- 47. In Kartha, K.K. (Ed). *Cryopreservation of Plant Cells and Organs*. CRC Press, Boca Raton.

Meskimen, G., and Francis, J. F. (Accessed on 26th January 2009). *Eucalyptus grandis* Hill Ex Maiden- Rose Gum *Eucalyptus*.
http://www.na.fs.fed.us/spfo/pubs/silvics_manual/volume_2/eucalyptus/grandis.htm

Milborrow, B. V. (1981). Absciscic acid and other hormones. In Paley, G., and Aspinall, D. (Eds). *The Physiology and Biochemistry of Drought Resistance in Plants*. Academic Press, Australia.

Mills, D., Zhang, G., Benzioni, A. (2001). Effect of different salts and of ABA on growth and mineral uptake in Jojoba shoots grown *in vitro*. *Journal of Plant Physiology* 158, 1031-1039.

Mlambo, C., K. (2004). The effect of physical desiccation for cryopreservation of *Eucalyptus* axillary buds cultured *in vitro*. Master of Science Dissertation, Department of Animal, Plant and Environmental Sciences, University of Witwatersrand, Johannesburg, South Africa.

Moons, A., Gielen, J., Vandekerckhove, J., Van Der Straeten, D., Gheysen, G., Van Montagu, M. (1997). An Absciscic acid and salt stress responsive rice cDNA from a novel plant gene family. *Planta* 202, 443- 454.

Moore, J.P., Vire- Gibouin, M., Farrent, J.M., Dirouich, A. (2008). Adaptations of higher plant cell walls to water loss: drought vs. desiccation. *Physiologia plantarum* 134, 237-245.

Morabito, D., Mills, D., Prat, D., and Dizengremel, P. (1994). Response of clones of *Eucalyptus microtheca* to NaCl *in vitro*. *Tree Physiology* 14, 201-210

Morabito, D., Jolivet, Y., Prat, D., and Dizengremel, P. (1996). Differences in the physiological response of two clones of *Eucalyptus microtheca* selected for their salt tolerance. *Plant Science* 114, 129-139.

Mundy, J., Nielsen, H. B., and Brodersen, P. (2006). Crosstalk. *Trends in Plant Science* 11, 63-64.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays of callus cultures. *Physiologia Plantarum* 15, 473-497.

Mycock, D. J., Berjak, P., Pammenter, N. W., and Vertucci, C. W. (1997). Cryopreservation of somatic embryoids of *Phoenix dactylifera* (pp75- 82). In Ellis, R. H., Black, M., Murdoch, A. J., and Hong, T. D. (Eds). *Basic and applied aspects of seed biology*. Kluwer Academic Publishers. Great Britain.

- Mycock, D. (1999). Addition of calcium and magnesium to a glycerol and sucrose cryoprotectant solution improves the quality of plant embryo recovery from cryostorage. *Cryoletters* 20, 77-82.
- Mycock, D. J., Blakeway, F. C., and Watt, M. P. (2004). General applicability of *in vitro* storage technology to the conservation and maintenance of plant germplasm. *South African Journal of Botany* 70, 31-36.
- Padayachee, K. (2000). The development of medium term and long term storage protocols for *Eucalyptus grandis*. MSc thesis. University of Witwatersrand, Johannesburg.
- Padayachee, K., Watt, M. P., Edwards, N., Mycock, D.J. (2008). Physiological responses of *Eucalyptus in vitro* axillary buds to cryopreparative desiccation and osmotic preculture: Effects of abscisic acid. *South African Journal of Botany*. 74, 639-646.
- Panis, B., and Lambardi, M. (2005). Status of cryopreservation technologies in plants (crops and forest trees). *The Role of Biotechnology*, pp 43- 54, Villa Gualino, Turin, Italy.
- Pastori, G. M., and Foyer, C. H. (2002). Common components, networks and pathways of cross tolerance to stress. The central role of 'redox' and abscisic acid mediated controls. *Plant Physiology* 129, 460-468.
- Paulet, F., Engelmann, and Glaszmann, J. C. (1993). Cryopreservation of apices of *in vitro* plantlets of sugarcane (*Saccharum* sp. hybrids) using encapsulation/ dehydration. *Plant Cell Reports* 12, 525-529.
- Pence, V. C., Dunford, S. S., and Redella, S. (2005). Differential effects of abscisic acid on desiccation tolerance and carbohydrates in three species of liverworts. *Journal of Plant Physiology* 162, 1331-1337.
- Phillips, J. R., Oliver, M. J., and Bartels, D. (2002). Molecular genetics of desiccation and tolerant systems (pp 319-342). In Black, M., and Pritchard, H.W (Eds). *Desiccation and Survival in Plants- Drying Without Dying*. CABI Publishing, UK.
- Pierce, B. T., and Verry, S. D. (2001). Five year results from a site by clone interaction trial series of *Eucalyptus grandis* in summer rainfall areas of South Africa. *Forestry Genetics for the Next Millennium*, pp186-192. Proceedings of the IUFRO Working Party, Durban, South Africa.
- Purohit, S., Kumar, P., Larloraya, M., Bharti, S., and, Loloraya, M. M. (1992). ABA- induced 'lipid melting' and its reversal by umbiferone in the plasmalemma of guard cells protoplast: a breakthrough in plant hormone – receptor binding and hormone action. *Biochemical and biophysical research community* 186, 652-658.
- Qadir, M., Tubeileh, A., Akhtar, J., Larbi, A., Minhas, P. S., and Khan, M. A. (2008). Productivity enhancement of salt- affected environments through crop diversification. *Land Degradation and Development* 19, 429-453.

- Raymond, C. A. (2002). Genetics of *eucalyptus* wood properties. *Annals of Forestry Science* 59, 525-531.
- Rock, C. D., and Quatrano, R. S. (1995). The role of hormones during seed development. (pp 671-197). In Davies, P.J. (Ed). *Plant hormones: physiology, biochemistry and molecular biology*. Kluwer. Dordrecht, The Netherlands.
- Ross, J., and O'Neill, D. (2001). New interactions between classical plant hormones. *Trends in Plant Science* 6, 2-4.
- Sairam, R. K., and Tyagi, A. (2004). Physiology and molecular biology of salinity stress tolerance in plants. *Current Science* 86, 407-421.
- Schroeder, J. I., Allen, G. J., Hugouvieux, V., Kwak, J. M., Waner, D. (2001). Guard cell signal transduction. *Annual Review of Plant Physiology & Plant Molecular Biology* 52, 627-658.
- Shalata, A., and Neumann, P. M. (2001). Exogenous ascorbic acid (vitamin C) increases resistance to salt stress and reduces lipid peroxidation. *Journal of Experimental Botany* 52, 2207-2211.
- Sharma, S. K., and Ramamurthy, V. (2000). Micropropagation of 4- year old elite *Eucalyptus tereticornis* trees. *Plant Cell Reports* 19, 511-518.
- Shinozaki, K., and Yamaguchi- Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: differences and cross talk between two stress signaling pathways. *Current Opinion in Plant Biology* 3, 217-223.
- Shinozaki, K., Yamaguchi- Shinozaki, K., and Seki, M. (2003). Regulatory network of gene expression in the drought and cold stress response. *Current Opinion in Plant Biology* 6, 410-417.
- Shinozaki, K., and Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany* 58, 221-227.
- Skriver, K., and Mundy, J. (1990). Gene expression in response to abscisic acid and osmotic stress. *Plant Cell* 2, 503-512.
- Sreedhar, L., Wolkers, W. F., Hoekstra, F. A., and Bewley, J. D. (2002). *In vivo* characterization of the effect of abscisic acid and drying protocols associated with the acquisition of desiccation tolerance in *Alfalfa (medicago sativa L.)* somatic embryos. *Annals of Botany* 89, 391-400.
- Stewart, P., Talyor, M., and Mycock, D. (2001). The sequence of the preparative procedures affects the success of cryostorage of Cassava somatic embryos. *CryoLetters* 22, 35-42.
- Sun, D., and Dickinson, G. R. (1993). Response of salt stress of 16 *Eucalyptus* species, *Grevillea robusta*, *Lophostemon confertus* and *Pinus caribea var hondurensis*. *Forest Ecology and Management* 60, 1-14.

- Swamy, P. M., and Smith, B. N. (1999). Role of abscisic acid in plant stress tolerance. *Current Science* 76, 1220-1227.
- Sweby, D., Hockett, B. I., and Watt, M. P. (1994). Effects of nitrogen nutrition on salt stressed *Nicotiana tabacum* var. *Samsun* *in vitro* plantlets. *Journal of Experimental Botany* 45, 995-1008.
- Thokoane, N. L. (1999). *The development of short- to medium- and long- term germplasm storage protocols for Eucalyptus Spp.* MSc thesis. University of Natal.
- Tunnacliffe, A., and Wise, K. J. (2007). The continuing conundrum of the LEA proteins. *Naturwissenschaften* 94, 791-812.
- Tunnacliffe, A. (2008). LEAP Year 2008: LEA Proteins coming of age. *South African Association of Botanists*, pp 40. Drakensburg, South Africa.
- Walters, C., Farrant, J. M., Pammenter, N. W., and Berjak, P. (2002). Desiccation stress and damage, pp 263-291. In: Black, M., and Pritchard, H. W. (Eds). *Desiccation and Survival in Plants- Drying Without Drying*. CABI Publishing, United Kingdom.
- Wang, X. J., Loh, H. H., and Sun, W. Q. (2002). Drying rate and dehydrin synthesis associated with abscisic acid- induced dehydration tolerance in *Spathoglottis plicata* orchidaceae protocorms. *Journal of Experimental Botany* 53, 551-558.
- Watt, M. P., Duncan, E. A., Ing, M., Blakeway, F. C., and Herman, H. (1995). Performance of macropropagated and micropropagated *Eucalyptus* hybrids. *South African Forestry Journal*. 173: 17- 21.
- Watt, M. P., Mycock, D. J., Blakeway, F. C., and Berjak, P. (2000a). Applications of *in vitro* methods to *Eucalyptus* germplasm conservation. *Southern African Forestry Journal* 187, 3-10.
- Watt, M.P., Thokoane, N.L. Mycock, D.J. & Blakeway, F. (2000b). *In vitro* storage of *Eucalyptus* germplasm under minimal growth conditions. *Plant Cell, Tissue & Organ Culture* 61, 161-164.
- Watt, M. P., Blakeway, F. E., Mokotedi, M. E. O. and Jain, S. M. (2003). Micropropagation of Eucalypts, pp217-244. In: Jain, S. M., and Ishii, K. (Eds). *Micropropagation of woody trees and fruits*. Kluwer Academic Publishers, The Netherlands.
- Wesley- Smith, J., Walters, C., Berjak, P., and Pammenter, N. W. (2001). The Effect of two drying rates on the desiccation tolerance of embryonic axes of recalcitrant jackfruit (*Artocarpus heterophyllus* Lamk.) seeds. *Annals of Botany* 88, 653-664.
- Wilkens, C. P., and Dodds, J. H. (1983). Tissue culture conservation of woody species, pp 113-136. In Dodds, J. H (Ed). *Tissue Culture of Trees*. Avi Publishing Company, Connecticut.

Wise, K. J., and Tunnacliffe, A. (2004). POPP the question: what do LEA proteins do? *Trends in Plant Science* 9, 2004.

Withers, L. A. (1986). *In vitro* approaches to the conservation of plant genetic resources. In Withers, L. A., and Alderson, P. P. (Eds), *Plant tissue culture and its agricultural applications*, pp 261-273. Butterworth, London.

Withers, L. A. (1989). *In vitro* conservation and germplasm utilization. In Withers, L. A., and Alderson, P. P. (Eds), *Plant tissue culture and its agricultural applications*, pp 261-273. Butterworth, London.

Xiong, L., and Zhu, J. K. (2002). Molecular and genetic aspects of plant response to osmotic stress. *Plant, Cell, and Environment* 25, 131-139.

Xiong, L., and Zhu, J. (2003). Regulation of abscisic acid biosynthesis. *Plant Physiology* 133, 29-36.

Yokoi, S., Bressan, R. A., and Hasegawa, P.M. (2002). Salt stress tolerance of plants. JIRCAS Working Report. Pp25-33.

Yu, X. M., Griffith, M., Wiseman, S. B. (2001). Ethylene induces antifreeze activity in Winter Rye leaves. *Plant Physiology* 126, 1232-1240.

Zhu, J. K. (2001). Plant salt tolerance. *Trends in Plant Science* 6, 66-71.