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2013-04-22
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ABIOTIC STRESS CROSS-TOLERANCE IN *Eucalyptus grandis*

Does pre-exposure to chilling stress induce cross-tolerance to cryopreparative drying of *in vitro* shoots of *E. grandis*?

By

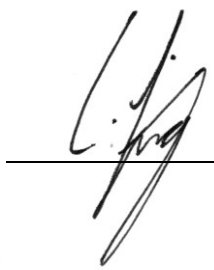
CHAO-HSUAN TING

A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

School of Animal, Plant and Environmental Sciences,

University of the Witwatersrand,

Johannesburg, South Africa



signed on the 24th day of October, 2012 in Johannesburg

DECLARATION

The experimental work described in this dissertation was carried out in the School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Johannesburg, South Africa, under the supervision of Dr Kershree Padayachee and co-supervision of Professor David J Mycock (School of Animal, Plant and Environmental Sciences, University of the Witwatersrand).

I declare that this Dissertation is my own original and unaided work, and has not been submitted in any form for any degree or diploma at any other tertiary institution. Any research work conducted by other investigators has been duly acknowledged in the following text, as evidenced by citations and references.



CHAO-HSUAN TING

24th day of OCTBOER, 2012 in JOHANNESBURG

DEDICATION

This dissertation is dedicated to my late mother,

Juling Shen (1956-2000),

for her sacrifices and perseverance for her children.

ACKNOWLEDGMENTS

I would like to extend my sincerest gratitude and appreciation to my supervisor Dr Kershree Padayachee and co-supervisor Prof David J Mycock, for their guidance, support and encouragement, and advice throughout the duration and completion of the project and this dissertation. I am truly grateful and humbled to have such wonderful and patient supervisors with such unprecedented wisdom, knowledge and experience in their respective fields during the course of this project.

To my brother and sister, thank you for your constant motivation and support, understanding, and especially encouragement for me to continue to pursue my research aspirations. To my father, thank you for your support, encouragement and commitment up to this point for my education. Also, thank you to Milton and Arabella for keeping me company during the synthesis of this Dissertation. Without you all, I could not have accomplished this endeavour.

Finally, I would like to thank the University of the Witwatersrand and the National Research Foundation for financial support, and Mondi Group (South Africa) for the plant material. I would also like to express my sincerest gratitude to Dr K Padayachee for the financial support provided to me and throughout this project.

ABSTRACT

In the forestry industry the requirement for the maintenance of a broad genetic base is integral to the success of breeding programmes such as those for *Eucalyptus grandis*, an important species to the South African forestry industry. Plant cryopreservation is an economical option to maintain such a genetic base, as it allows storage of vegetative materials at sub-zero temperatures, while maintaining juvenility. However, successful cryopreservation of this sub-tropical species has been restricted by its sensitivity to drying. As a consequence, the viability of material is reduced even before reaching the freezing stage. Despite this abiotic stress restriction, evidence of upstream 'cross-talk' implying downstream 'cross-tolerance' has suggested the possibility that cold acclimation may improve the tolerance responses towards dehydration stress by means of 'cross-acclimation'. It was therefore the aim of the study to understand some of the physiological and biochemical responses of *in vitro E. grandis* shoots to different non-freezing low (chilling) temperatures and exposure periods, and to establish an appropriate 'cross-acclimation' regime for the physical drying pre-treatment.

E. grandis shoot clusters (4-8 leaves and 2-5 axillary buds) were exposed to the chilling temperatures of 5°C, 10°C or 15°C for 1 or 3 days. The physiological and biochemical responses were evaluated, and thereafter the appropriate cold acclimation (or 'cross-acclimation') regime selected. The appropriate physical drying time was also selected for shoot clusters according to their physiological responses. When the appropriate regimes had been determined, the physiological and biochemical responses of shoot clusters treated consecutively to cold acclimation and then physical drying were evaluated. The physiological responses evaluated were water content, viability, and vigour (i.e. the number of visible axillary buds and shoots produced over 2 weeks). The biochemical responses measured were the concentrations of: 1) total soluble sugars, 2) starch, 3) phenolic acid, and 4) superoxide.

The data suggested that the appropriate cold acclimation regime was treatment at 10°C for 3 days. This was based on the accumulation of the high levels of phenolic acid (3.05 ± 0.09 mg GAE.g^{-1} FWS) and positive vigour responses (11.90 ± 0.60 visible axillary buds/week and 3.10 ± 0.20 visible shoots/week), compared with the other chilling temperature treatments. The appropriate drying time selected for shoot clusters was 80 min over activated silica gel to achieve a water content of 0.32 ± 0.04g water.g^{-1} FWS. In the dried material there were high levels of soluble sugars (47.65 ± 1.90%) and unknown components (48.10 ± 1.86%). Despite

these measured responses, viability of the shoots was impacted by drying, dropping to 88.9 ± 3.9%. When shoot clusters were pre-treated at 10°C for 3 days and then dried, viability of all (100%) the material was retained and the water content did not drop as low as with drying alone, dropping to 0.52 ± 0.05g water.g^{-1} FWS. The biochemical responses showed that tolerance was strongly dependent on a high proportion of soluble sugars (83.66 ± 1.48%) and then phenolic acid (3.77 ± 0.12%).

The study had confirmed that 'cross-acclimation' through means of cold acclimation (chilling pre-treatment at 10°C for 3 days) can induce 'cross-tolerance' towards physical drying, where osmotic adjustments and osmoprotection appeared to have been improved. It is therefore possible that this may have the potential to improve survival during the latter stages of the cryopreservation procedure, despite the higher retention of water in shoot clusters after drying.

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LIST OF SYMBOLS AND NOMENCLATURE

ABA	abscisic acid
~	approximately
ATP	adenine triphosphate
°C	degrees Celsius
cm	centimetres
cm^3	cubic centimetres
DNA	deoxyribonucleic acid
FC	Folin-Ciocalteau reagent
FWS	fresh weight of shoots
GAE	gallic acid equivalent
GLM	General Linear Model
GFG	glucose, fructose and galactose
$g.g^{-1}$	grams per grams
$g.l^{-1}$	grams per litre
≥	greater or equal to
>	greater than
HSD	honest significant difference
H	hours
<	less than
×	hybrid cross (breeding)
LN	liquid nitrogen
$l.mol^{-1}.cm^{-1}$	litres per molar per centimetre
MPa	mega Pascal
$\mu g.ml^{-1}$	micrograms per millilitre
$ml.l^{-1}$	millilitres per litre
$mg.ml^{-1}$	milligrams per millilitre
min	minutes
MAPK	mitogen-activated protein kinase
M	molar
mM	millimolar
MS	Murashige & Skoog nutrient formulation (1962)
nm	nanometres
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
no.	number
%	percentage
% (w/v)	percentage (weight per volume)
PGR(s)	plant growth regulator(s)
±	plus and/or minus
p	probability (sample)
PCD	programmed cell death
RFOs	raffinose-family oligosaccharides
REDOX	reduction-oxidation
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions rep minute
n	sample size
Shoot(s)	shoot cluster(s)

NaCl	sodium chloride
SE	standard error
10×	ten times magnification
TSS	total soluble sugars
UV	ultraviolet
WC	water content
Ψ	water potential

CHAPTER 1. GENERAL INTRODUCTION

1.1 Background

Hardwood trees of the *Eucalyptus* genus (eucalypts) have become an invaluable economic commodity of the forestry industry. These long-lived evergreen and roundwood trees include more than 600 species that are part of the angiosperm family Myrtaceae (Grattapaglia, 2008; Ladiges *et al.*, 2003). Together with the genera of *Pinus* and *Acacia*, eucalypts pre-dominate the fast-growing forestry plantations around the world, approximated to have produced about 20% of annual harvests (Christie, 2008). Of the estimated 50 million hectares (ha) of plantations around the globe, approximately 17.9 million ha are of eucalypts (Christie, 2008; Grattapaglia, 2008).

Eucalypts are favoured in the forestry industry for several characteristics. In particular, they are known to grow fast and have the ability to adapt to different environments (Grattapaglia, 2008; Padayachee, 2007). As a consequence, they have become distributed in most temperate and tropical regions (Grattapaglia, 2008; Padayachee, 2007). Specific species selected for forestry have uniform straight growth forms and valuable wood properties such as high fibre content, and are also easily managed through coppicing (Grattapaglia, 2008; Padayachee, 2007). Their main industry application is the production of high quality short-fibre pulp for paper and pulp industries (Christie, 2008; Durand-Creswell *et al.*, 1982; McComb & Bennet, 1986; Turnball & Pryor, 1984), where they represent more than 50% of the international market for pulp trade (FAO, 2007). Eucalypts also have widespread application as timber and sawn woods, a source of essential oils, wind and fire breaks, and rapid coverage of soil in preventing erosion (Christie, 2008; Durand-Creswell *et al.*, 1982; Grattapaglia, 2008). Apart from its conventional roles, eucalypts have also been considered in afforestation for carbon sequestration, and as a source of biochemicals and feedstock for bioenergy (Christie, 2008; Kamm *et al.,* 2006).

Of the many species of eucalypts, only a few have been selected for forestry purposes and established as plantations (Grattapaglia, 2008; Ladiges *et al.*, 2003). The main species are *E. grandis*, *E. globulus*, and *E. urophylla*; these together with their hybrids, account for about 80% of plantations (Grattapaglia, 2008). In the forestry industry, seed and vegetative propagation of *E. grandis* and *E. globulus* are predominant in tropical and sub-tropical regions, while *E. urophylla* dominate temperate regions (Grattapaglia, 2008). The majority of

breeding, biotechnological and silvicultural developments and advancements therefore have focused on these species and their hybrids (Grattapaglia, 2008; Padayachee *et al.*, 2009). The priorities of such programmes include improving productivity, and genetically engineering and breeding more productive varieties adaptable to different environmental conditions, such as with *E. grandis* in South Africa (Grattapaglia, 2008; Padayachee *et al.*, 2009).

In South Africa, *E. grandis* is one of the most dominant hardwood species in commercial forestry (Malan, 1995; Padayachee, 2007; Poynton & SARCCUS, 1979). However, this species is intolerant of adverse environmental conditions such as drought and cold (Naidoo *et al.*, 2006; Padayachee, 2007; Padayachee, 2000; Poynton & SARCCUS, 1979). Thus, the propagation of this species makes it an unsuitable candidate for many marginal sites that are prone to drought, cold and frost (Padayachee, 2007). To overcome this constraint, tree improvement programmes have been established for *E. grandis* to develop suitable candidates for such sites (Padayachee, 2007; Poynton & SARCCUS, 1979). For example, the hybridisations of pure *E. grandis* with other species that are more tolerant of drought (e.g. *E. camaldulensis*) and frost (e.g. *E. nitens*) (Padayachee, 2007). Through hybridisations with species that are less suited for industry requirements, the progeny reproduced inherit intermediate characteristics of both the sensitive and tolerant species and therefore are able to grow successfully on sites that are marginal and unsuitable for the pure species (Padayachee, 2007). Thus, tree improvement programmes are integral in achieving forestry demands. However, without the maintenance of a broad genetic base of these species and their hybrids (genetic conservation of eucalypts) such hybridisation practices and breeding for meeting specific industry criteria would not be possible (Padayachee *et al.*, 2009; Watt *et al.*, 2000a).

1.2 Tree improvement and genetic conservation in South Africa

In South African tree improvement programmes, extensive selection practices for a superior variety coupled with intensive clonal propagation have been successful in increasing productivity and improving wood qualities, but the danger of this is that it can result in reduced genetic variability of the selected and propagated materials (Padayachee *et al.*, 2009; Watt *et al.*, 2000a). The subsequent consequences of reduced genetic variability would increase the susceptibility of plantations to environmental fluctuations and pathogens (Padayachee *et al.*, 2009). To address this challenge, the review by Padayachee *et al.* (2009) described the employment of strategies involving the maintenance of disparate genetic populations. The underlying purpose of these strategies is ultimately the genetic conservation

of *Eucalyptus* germplasms, which provides a resource of broad genotypes for breeders to meet the ever changing demands in commercial forestry (Padayachee *et al.*, 2009; Watt *et al.*, 2000a, b).

Commercial forestry in South Africa employs a variety of *ex situ* approaches for genetic conservation (Watt *et al.*, 2000a). These approaches include the establishment of stands of seed orchards, and storage of pollen and seeds (Padayachee *et al.*, 2009; Watt *et al.*, 2000ab). There are also the more active vegetative approaches, which include the establishment of clonal hedges for selected genotypes, and the maintenance of containerised rooted-cuttings in greenhouses and hydroponic systems (Watt *et al.*, 2000ab). However, these conventional approaches have their limitations. Seed orchards rely on manual pollination, since open pollination has the risk of inbreeding depression (Suitor *et al.*, 2008). Even under optimal storage conditions, viable seed and pollen collected from these orchards are subject to aging, thus limiting subsequent viability and longevity (Fantinatti & Usberti, 2011; Probert *et al.*, 2009; Rajjou & Debeaujon, 2008; Wheeler & McComb, 2006). Expansion of vegetative approaches and seed orchards are limited by the availability of suitable land, and in South Africa legislations on water and land usage for forestry are particularly stringent (Louw, 2006; Padayachee *et al.*, 2009; Watt *et al.*, 2000b). In addition, the management of these field-based germplasm depositories can be costly and labour-intensive, and remain susceptible to biotic (i.e. pathogens) and abiotic (e.g. drought and cold) stresses (Padayachee *et al.*, 2009; Watt *et al.*, 2000ab).

In order to address these challenges within the South African forestry industry, *in vitro* storage techniques were recommended and protocols were developed for specific species (e.g. *Eucalyptus grandis*) and explants to complement conventional approaches (Watt *et al.*, 2000a). These techniques involve the storage of small-sized explants under sterile and artificially controlled environmental conditions, which are integrated with the process of plant tissue culture (Watt *et al.*, 2000ab). Minimal growth storage is one of the *in vitro* techniques that had been well established for *E. grandis* shoots, roots and meristems, and involves the induction of slow states of growth (Padayachee, 2000; Watt *et al.*, 2000ab). However, this technique is only a medium-term solution for the maintenance of germplasm, since cultures are limited by their regeneration capacity with time, which is influenced by species, the type of explant, and the selected method to induce slowed growth (Gaspar *et al.*, 2002; Watt *et al.*, 2000a). Thus, cryopreservation has been considered as a viable long-term genetic conservation approach (Padayachee *et al.*, 2009).

1.3 Cryopreservation

Cryopreservation is a long-term *in vitro* storage technique that has been employed for a broad range of organisms, where specimens are maintained at ultra-low temperatures i.e. -196°C achieved with liquid nitrogen (Engelmann, 2004; Mycock *et al.*, 2004; Padayachee *et al.*, 2009) or in the vapour phase above liquid nitrogen (Berjak *et al.*, 2011). This technique has been acknowledged as a safe and cost-effective technique for the conservation of orthodox, recalcitrant and intermediate seed-producing species, and clonally propagated species, such as for *E. grandis* (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Mycock *et al.*, 2004; Padayachee *et al.*, 2009). Theoretically, it is believed that under such a storage temperature, the normal metabolic activities are effectively halted within cells, therefore permitting an unlimited storage period (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Padayachee *et al.*, 2009).

The success of cryopreservation has been seen with undifferentiated cultures such as cell suspensions and calli but these materials are prone to genetic variability (Bajaj, 1995; Engelmann, 1991; Engelmann, 2004; Withers, 1988). In contrast, differentiated structures (e.g. shoot tips and meristems) are considered to be genetically stable and already in a developed state primed for growth, thus some differentiated structures have also been successfully cryopreserved, like the apices of cold-tolerant plant species (Bajaj, 1995; Engelmann, 1991; Engelmann, 2004; Withers, 1988). Despite the appeal of this technique, cryopreservation has a very stringent set of requirements for materials, accomplished through a complex process that can be stress inducing and extremely influential on the subsequent viability of materials (Bajaj, 1995; Berjak *et al.*, 2011; Padayachee *et al.*, 2008 and 2009).

1.3.1 Requirements of cryopreservation

The requirements for the optimum water content and the size of the material are of particular importance for successful cryopreservation (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Mycock *et al.*, 2004). This is because many candidate materials (cell suspensions, calluses, shoot tips, embryos etc.) are highly hydrated, and thus are susceptible to freezing injury, since most of these are inherently freezing-intolerant (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Mycock *et al.*, 2004; Padayachee *et al.*, 2009). The amount of ice crystal formation within the material is governed by the content of water, and this is dependent on the size and type of the material, which also determines the rate of freezing and thawing (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Mycock *et al.*, 2004). By artificially removing cellular

water and minimizing the size of cryopropagules, the component cells/tissues would be protected from damage caused by crystallization of intracellular water into ice (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Mycock *et al.*, 2004; Padayachee *et al.*, 2009). Thus, in principle artificial dehydration may prevent freezing injury and maintain post-thaw viability (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Mycock *et al.*, 2004; Padayachee *et al.*, 2009). Many of the cryopreparative procedures incorporate the principle of artificial dehydration for cryoprotection, but the methods used to achieve dehydration (chemical or physical) are different and in themselves often injurious (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008). The so-called cryoprotectants are a heterogenous group of chemical compounds that osmotically remove water from cells/tissues (e.g. sucrose) to sites of extracellular ice nucleation (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008). Alternatively, cryoprotectants can penetrate cells (e.g. glucose and proline) and replace intracellular water (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008). Physical drying is achieved in a number of ways e.g. equilibrium drying to specific relative humidity, drying in a stream of sterile air, and drying over activated silica gel (Padayachee *et al.*, 2009).

Artificial dehydration, chemical or physical, aims to remove the bulk of cellular water and concentrate the contents of the cells, thereby increasing osmotic potential. It has been suggested that dehydrating samples to water content levels between 10 and 20% of the fresh weight would be optimal for survival when frozen (Berjak *et al.*, 1989; Engelmann, 2004). The desired effect of artificial dehydration is to promote a physical state of vitrification within cells when freezing is underway, and this could trigger freeze-avoidance mechanisms (Gonzalez-Arnao *et al.*, 2008). Vitrification involves the physical transition of the remaining liquid phase in the cytoplasm and organelles to an amorphous glassy state, which is promoted at a specific glass-transition temperature (Engelmann, 1991; Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008). The high viscosity of the vitrified state virtually stops all chemical reactions requiring molecular diffusion of water, and thus the material can be maintained under this stable condition for a long period (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008). The advantages of this vitrified state include preventing tissue collapse, and alterations in pH and solute concentration (Gonzalez-Arnao *et al.*, 2008).

Vitrification can also be achieved by freezing the material fast enough to preclude ice crystal formation, which sometimes can be achieved by direct immersion in liquid nitrogen, (Engelmann, 2004; Engelmann, 1991; Gonzalez-Arnao *et al.*, 2008; Sakai, 1995). However, prior to immersion, the material undergoes extensive pre-treatment of chemical osmotic

dehydration or physical drying (Engelmann, 1991; Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Sakai, 1995). There are seven procedures identified that incorporate the principle of dehydration in promoting vitrification (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Table 1). These vitrification-based methods have shown some success with the cryopreservation of differentiated structures, such as embryos and shoot-tips (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Sakai, 1995).

Table 1. A brief summary of the seven vitrification-based methods

Procedure	Description	Material Type	References
Pre-growth	• Material cultivated with cryoprotectant(s)	Meristems	Engelmann, 1991 and 2004
Physical Drying	• Drying by flow of sterile compressed air, silica gel or ultra-rapid drying for a defined time • 10-30% Water content, fresh weight basis	Embryos Embryonic axes	Engelmann, 2004 Padayachee *et al.*, 2008 Padayachee *et al.*, 2009 Padayachee, 2000
Vitrification	• Firstly, "loading solution" exposure to improve osmotic tolerance e.g. 2M glycerol + 0.4M sucrose (Matsumoto *et al.*, 1994) • Secondly, pre-treatment with Plant Vitrification Solution e.g. PVS: glycerol, ethylene glycol, dimethyl sulfoxide, and sucrose (Sakai *et al.*, 1990)	Apices Cell suspensions Embryogenic tissues Somatic embryos	Engelmann, 2004 Gonzalez-Arnao *et al.*, 2008
Encapsulation-dehydration	• Preculture on high sucrose concentration, and dried to 20% water content • Encapsulation of material in calcium-alginate beads • Simultaneous encapsulation and pre-growth with glycerol and sucrose	Apices Cell suspensions Somatic embryos	Engelmann, 2004 Gonzalez-Arnao *et al.*, 2008
Encapsulation-vitrification	• Encapsulation-dehydration and vitrification combined • Vitrification occurs after encapsulation-dehydration	Apices	Engelmann, 2004 Gonzalez-Arnao *et al.*, 2008
Pre-growth-dehydration	• Pre-growth in presence of cryoprotectant(s) • Dehydration by drying	Stem segments Polyembryos Zygotic embryos	Engelmann, 2004 Gonzalez-Arnao *et al.*, 2008
Droplet-vitrification	• Vitrification and droplet freezing combined • Droplet freezing: material frozen with droplet of cryprotectant(s)	Apices	Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008

1.3.2 Biological stress caused by the process of cryopreservation

The process of cryopreservation can involve the following successive stages (Fig 1): choice of material, pre-treatment, freezing, storage (usually in liquid nitrogen), thawing, post-treatment and recovery (Bajaj, 1995; Engelmann, 1991; Withers, 1988). The specificity of the procedures of each stage can vary depending on the material of choice and the species, where each stage must be specifically adapted and optimised (Bajaj, 1995; Withers, 1988). These successive stages can cause damage on the material itself, which would influence its subsequent viability (Chen & Wang, 2002; Engelmann, 1991; Padayachee *et al.*, 2008; Seo *et al.*, 2007; Sershen *et al.*, 2007; Whitaker *et al*., 2010).

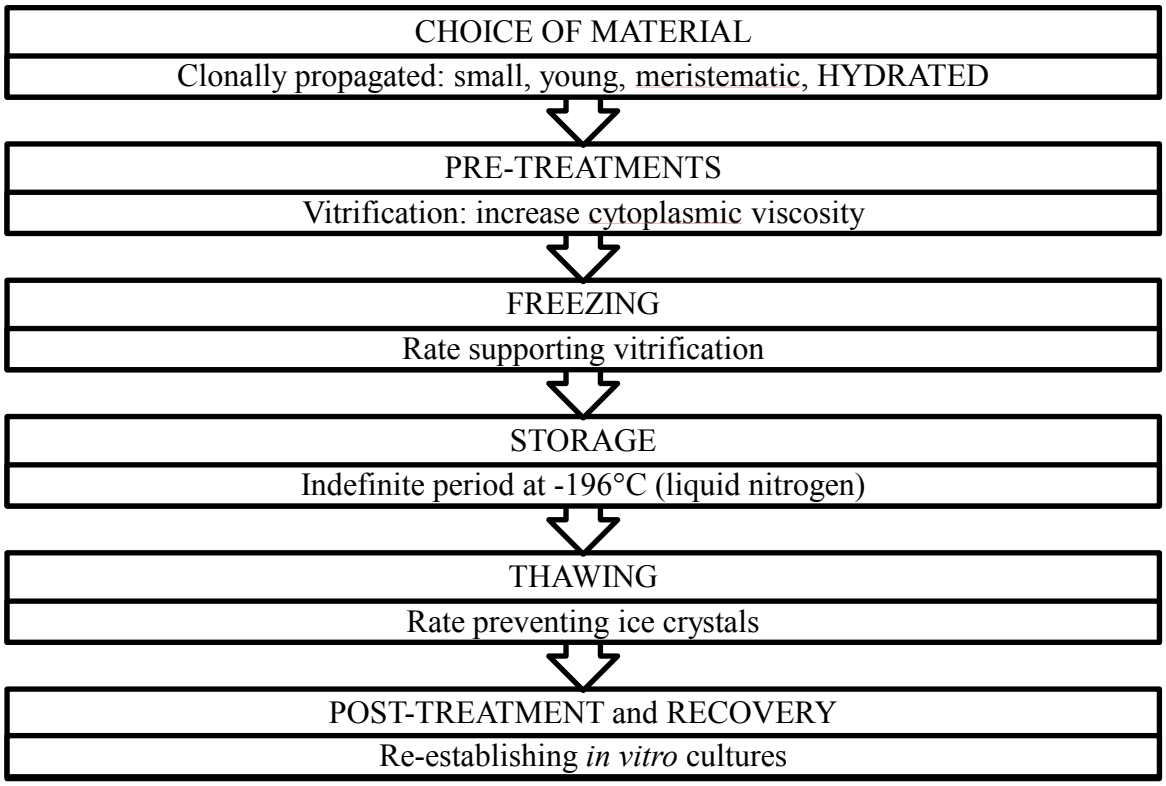


Fig 1. Possible steps in the process of cryopreservation for clonally propagated material (Bajaj, 1995; Engelmann, 1991)

1.3.2.1 Choice of material

Preferably, the plant cryopropagule must be young, meristematic and as small as possible (Engelmann, 1991; Mycock *et al.*, 2004). However, the isolation of materials with such characteristics (e.g. axillary buds, shoot-tips, meristems and embryonic axes) require careful excision from the parent source (Bajaj, 1995; Engelmann, 2004; Engelmann, 1991; Withers, 1988; Whitaker *et al*., 2010). This excision process can wound the material, and stimulate the production of potentially harmful reactive oxygen species (ROS; superoxides, hydrogen peroxide and hydroxyl radicals etc.) that cause oxidative stress (Gaspar *et al.*, 2002; Whitaker *et al*., 2010).

1.3.2.2 Pre-treatments

The majority of the vitrification-based procedures require the use of cryoprotectants (e.g. sucrose, sorbitol, mannitol, polyethylene glycol, and dimethylsulfoxide), which have been extensively applied in order to achieve artificial dehydration (e.g. Engelmann, 1991, Gonzalez-Arnao *et al.*, 2008 and Padayachee *et al.*, 2009). Cryoprotectants can be used in combination or individually, but all principally function in the removal of some cellular water (Engelmann, 1991; Gonzalez-Arnao *et al.*, 2008). However, much of the evidence suggests that cryoprotectants can directly cause injury (e.g. plasmolysis) because of their chemical toxicity and by causing osmotic stress (Chen & Wang, 2002; Padayachee, 2000). For example, dimethylsulfoxide concentrations greater than 15% (v/v) are known to be toxic to certain plant tissues (Tokatli & Ademir, 2010), since high concentrations of dimethylsulfoxide may cause biomembrane damage and even affect protein structure and the genetic regulation of cell differentiation (Yu & Quinn, 1994).

An alternative to the use of cryoprotectants is physical drying (Section 1.3.2, Table 1). It has been suggested that the utilization of activated silica gel is a more suitable choice for drying of delicate materials such as *in vitro* axillary buds of *E. grandis* (Padayachee *et al.* 2008). The degree of dehydration by drying depends on the rate of water loss (related to drying time), and this can affect the overall viability of materials (Padayachee *et al.* 2008; Seo *et al.*, 2007; Sershen *et al.*, 2007; Varghese *et al.*, 2011). If there is insufficient removal of water by drying, this can lead to injury during freezing because of intracellular ice formation (Chen & Wang, 2002), whereas excessive drying can result in destabilisation of membranes (Padayachee *et al.*, 2008) and increased secondary oxidative stress (Sershen *et al.*, 2012; Whitaker *et al*., 2010).

It has been found that viability and water content of isolated *in vitro E. grandis* axillary buds significantly decreased as physical drying time over activated silica gel was prolonged (Padayachee *et al.* 2008). Although the favourable cellular water content level for vitrification was attained (Section 1.3.2), longer drying periods resulted in detectable ultra-structural damages in cells and death of cells (Padayachee *et al.* 2008). Padayachee *et al.* (2008) found that drying had caused lethal damage, characterised by severe plasma membrane separation and lack of sub-cellular structure; moderate damage was characterised by slight plasma membrane withdrawal and de-differentiation of mitochondria.

The extent and rate of dehydration are therefore important in the preparation of *in vitro* tissues for cryopreservation, similar requirements have been found with other plant tissues (Seo *et al.*, 2007; Sershen *et al.*, 2007; Whitaker *et al*., 2010). Embryonic axes isolated from amaryllid species that produce recalcitrant seeds, for example, have shown ≥50% survival when water content was reduced to between 0.25 and 0.05 g (water)/g (dry weight), even though viability is negatively affected below 0.45 g/g (Sershen *et al.*, 2007). In this regard, embryonic axes of *Trichilia dregeana* Sond (recalcitrant) dried to water contents of 39% and 21% and cryopreserved at those water contents have also shown elevated production of ROS which are detrimental to material survival (Whitaker *et al*., 2010).

1.3.2.3 Freezing and thawing

Rapid freezing (*x* to *y* degrees per unit) and controlled slow freezing (*a* to *b* degrees per unit) in cryopreservation are the methods that have been employed to achieve the necessary artificial freezing conditions that would promote vitrification and preclude ice crystal formation (Bajaj, 1995; Engelmann, 1991; Engelmann, 2004). During thawing, cellular damage can also occur because of ice crystal formation (Padayachee, 2000). The transition of ice structures from vitreous to crystalline and the re-crystallisation through the fusion of micro-crystals formed via freezing can occur during the process, so it is important to thaw at a rate which bypasses the occurrence of micro-crystals that would lead to crystallisation (Engelmann, 2004; Padayachee, 2000; Withers, 1988).

1.3.2.4 Post-treatment and recovery

Post-treatment is especially necessary for the removal of cryoprotectants from materials, and generally this relies on a progressive removal process that is based on dilution and diffusion (Chen & Wang, 2002; Engelmann, 1991). Recovery involves the culturing of materials on to

plant tissue culture medium containing additional plant growth regulators (PGRs) that induce re-growth and regeneration, and may assist in the selection of viable materials (Bajaj, 1995; Engelmann, 1991; Withers, 1988). Both removal of cryoprotectants and recovery involves the transfer of cryopreserved materials on to tissue culture medium that has low osmotic potential (Engelmann, 1991). This means that rehydration (influx of water into cells) of the material may occur too rapidly, which can cause cellular damage and impact survival, since protective mechanisms may not yet be operational (Oliver *et al.*, 1997). Sometimes, successive transfers of the material to lower osmotic potential media are necessary as to attenuate the osmotic shock (Engelmann, 1991). In addition, extracellular bursts of ROS are also displayed upon this rehydration during post-treatment and recovery (Sershen *et al.*, 2012; Whitaker *et al.*, 2010), where ROS is believed to trigger cell death (Van Breusegem & Dat, 2006).

1.3.3 Limitations of cryopreservation for *E. grandis*

Cryopreservation has been successful applied to a range of species especially those of temperate origin, those that produce orthodox seeds, and those that develop dormant buds (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Mycock *et al.*, 2004; Padayachee *et al.*, 2009). However, the technique is not without its obstacles, especially evident with *E. grandis* (Padayachee *et al.*, 2008). Vegetative and differentiated structures considered for cryopreservation are naturally hydrated, therefore susceptible to the formation of crystalline-ice, whenever cellular water is exposed to freezing temperatures (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Mycock *et al.*, 2004; Padayachee *et al.*, 2009). If the water is not properly removed to an acceptable level, these ice crystals can cause mechanical damage and freeze-induced desiccation, which are detrimental to overall viability (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Padayachee *et al.*, 2009; Schulze *et al.*, 2005b).

Furthermore, there is enormous variability between plant species in response to cryopreservation, especially to that of the abiotic stresses dehydration, chilling, and freezing (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008). In this regard, temperate plants may be evolutionary more tolerant than sensitive tropical and sub-tropical species (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008). Cryopreservation of eucalypts of temperate origin has been successful, whereas various propagules of sub-tropical *E. grandis* have not survived the process, despite some success with calli that are prone to genetic variability and hence are not appropriate for conservation purposes or clonal forestry (Padayachee *et al.*, 2009; Padayachee *et al.*, 2008; Watt *et al.*, 2000a). The natural sensitivity of *E. grandis* to abiotic stresses has

limited cryopreservation success of *in vitro* axillary buds, because even prior to freezing, the pre-treatment of physical drying decreased viability (Padayachee *et al.*, 2009; Padayachee *et al.*, 2008). Thus, if the viability of *in vitro E. grandis* axillary buds cannot be assured during the important pre-treatment stage, the likelihood of surviving the remaining cryopreservation stages is diminished.

The many manipulations during the cryopreservation process that prepare differentiated structures, such as embryonic axes or isolated axillary buds, for artificial dehydration and freezing, may lead to subsequent failure in recovery of materials (Whitaker *et al.*, 2010). This in part is due to the cumulative harmful effects of prolonged or repeated extracellular bursts of ROS throughout each stage of the process (Whitaker *et al.*, 2010). This cumulative oxidative stress will have deleterious effects on the material and limit success (Whitaker *et al.*, 2010), because the intensity of the secondary stress may be further escalated.

This has been a particular challenge for materials from sub-tropical and tropical species, such as *E. grandis*, that are recalcitrant to cryopreservation. However, with an improved understanding of fundamental stress-tolerance mechanisms and sensitivities at the genomic, proteomic and metabolic levels, further developments in adaptations and optimizations would be complemented (Hirayama & Shinozaki, 2010; Padayachee *et al.*, 2009). Such understanding can be incorporated into the cryopreservation process to improve tolerance against the abiotic stresses encountered, beginning from the pre-treatment stage, to improve the likelihood of survival (Hirayama & Shinozaki, 2010; Padayachee *et al.*, 2009). However, it is equally important to have an in depth understanding of the consequential effects of these abiotic stresses on the plant before a comprehensive understanding of the fundamental stress-tolerance mechanisms in plants can be achieved.

1.4 Abiotic stress

There are a variety of abiotic stresses that are experienced by plants, such as that of salinity, water availability, and temperature extremes (Tuteja, 2009). However, the abiotic stresses of low temperature (chilling and freezing) and water availability (dehydration and desiccation) are common during the process of cryopreservation (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008; Padayachee *et al.*, 2009). Not only are these two types of stresses experienced individually but, like in the natural environment (Mittler, 2006), can also be experienced in combination at varying degrees of intensity. Thus, the continuous fluctuations and changes in

the natural environment are similarly mimicked by the artificial environmental changes brought about by the procedures during each stage of the cryopreservation process.

These environmental changes, be they artificial or natural, are factors determining overall plant development and survival (Gaspar *et al.*, 2002; Kranner *et al.*, 2010). This would undoubtedly trigger and induce subsequent reactions and responses to allow for the continued development and survival under the new environments, but could also equally result in unfavourable effects that could counteract growth and survival (Gaspar *et al.*, 2002; Kranner *et al.*, 2010).

1.4.1 Strain and stress concept

The terminologies of 'strains' and 'stresses' are often used to differentiate categorically the extent of the action and effect of the external environmental pressures on plants. However, the definitions of these two terms in the context of various studies have resulted in some ambiguity and misinterpretations. Thus, it is important that the definitions of 'strain' and 'stress' are understood in the context of this study. The first meaning of the term 'stress' in plants has been defined as unfavourable conditions and fluctuations in the present environment be it biotic or abiotic (Gaspar *et al.*, 2002; Kranner *et al.*, 2010; Levitt, 1980a; Nilsen & Orcutt, 1996). Thus, 'stress' is an environmental pressure that is known as a 'stress factor' or 'stressor' (e.g. dehydration and chilling), which can influence the state of metabolic equilibrium in plants (Gaspar *et al.*, 2002; Kranner *et al.*, 2010; Schulze *et al.*, 2005a; Levitt, 1980a; Nilsen & Orcutt, 1996). However, the extent of the influence by the 'stress' depends on the duration and intensity of the exposure (Gaspar *et al.*, 2002; Kranner *et al.*, 2010; Levitt, 1980a; Nilsen & Orcutt, 1996). This may not be limited to only an individual 'stress' but could apply equally to 'stresses' occurring simultaneously, as is experienced in the natural environment (Gaspar *et al.*, 2002; Kranner *et al.*, 2010; Levitt, 1980a; Nilsen & Orcutt, 1996).

The duration and intensity of the exposure to the 'stress' are the distinguishing factors between a plant experiencing a 'stress' or 'strain', where both results in alterations and changes of the plant state of metabolic equilibrium (Gaspar *et al.*, 2002; Schulze *et al.*, 2005a; Levitt, 1980a; Nilsen & Orcutt, 1996). In the context of this study, the second meaning of 'stress' is defined as a physical manifestation of death in plant cells and tissues, but that does not mean the overall plant does not survive or remain viable (Gaspar *et al.*, 2002; Schulze *et al.*, 2005a; Levitt, 1980a; Nilsen & Orcutt, 1996). For example, when stress-triggered senescence and browning occur in leaves (regarded as symptomatic indicators of cell death),

the symptoms may be localised and regional to parts of the plant due to apoptosis or necrosis (Foyer & Noctor, 2005; Steyn *et al.*, 2002). Thus, the large areas of regional and localised death may not affect the plants' overall viability, as long as the duration and intensity of the 'stress' does not exceed the overall whole plant systems' natural ability to cope (Gaspar *et al.*, 2002; Kranner *et al.*, 2010; Schulze *et al.*, 2005a). In contrast, a duration and intensity of 'stress' that does not result in death of plant cells and tissues is defined as 'strain' (Gaspar *et al.*, 2002; Kranner *et al.*, 2010; Levitt, 1980a; Nilsen & Orcutt, 1996). In such instances, 'strain' can result in the symptom of callus formation, which is common during growth under *in vitro* conditions (Gaspar *et al.*, 2002).

1.4.2 Dehydration and desiccation

When transpiration rate exceeds overall water uptake, water deficits are experienced by plants (Bray, 1997). Water deficits are experienced in the natural environment when there are occurrences of droughts; and in the context of cryopropagules it is experienced during the pre-treatment stages of removing cellular water, such as by physical drying (Bray, 1997; Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Padayachee *et al.*, 2009). However, these stress factors, can encompass varying intensities of water loss, which are regarded as dehydration or desiccation. Dehydration is an overall decline of water in the protoplasm (~0.3g water/g dry weight), thus it is the loss of bulk cellular water from the cytoplasm and organelles (Bray, 1997; Wood, 2005). Desiccation, on the other hand, goes further than dehydration in that there is a loss of water from the protoplasm where hydration shells of membranes and proteins of cells are gradually lost (Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Wood, 2005).

Dehydration and desiccation can cause plant cells to experience changes in solute concentrations, which in turn can alter water potential (Ψ) gradients (Bray, 1997; Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Wood, 2005). This would influence cell volume, membrane shape and cell turgidity (Beck *et al.*, 2007; Bray, 1997; Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Wood, 2005). In extreme cases, this can disrupt membrane integrity and lead to the breakdown of proteins and sub-cellular compartments (e.g. organelles), essential in normal metabolic functioning (Beck *et al.*, 2007; Bray, 1997; Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Wood, 2005). Membranes and proteins are integral structures for overall cell functioning because they act as important compartment, support structures and components for metabolic pathways (e.g. respiration and photosynthesis) as well as important barriers in maintaining homeostasis (Bray, 1997; Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Wood, 2005).

The formation of ATP, for example, requires a proton motive force, which is maintained by the mitochondrial double membrane and proteins directly and indirectly associated with this membrane for electron transport (Willey *et al.*, 2008). The chloroplast grana structures composed of stacked thylakoids housing chlorophyll pigments are also another example, which are vital for capturing light energy (Fernandez & Strand, 2008; Toldi *et al.*, 2009; Willey *et al.*, 2008). Thus, disruption of membrane integrity, proteins and sub-cellular compartments by dehydration and desiccation would impede plant function and if too severe can lead to cell and tissue death.

Furthermore, high water deficits (desiccation) results in the removal of the hydration effect (Beck *et al.*, 2007; Hoekstra *et al.*, 2001). This is central for the assembly of phospholipids into membranes and maintenance of multi-functional biological membranes, as well as for the structural conformation of proteins (Beck *et al.*, 2007; Hoekstra *et al.*, 2001). The loss of membrane integrity can also result in the escalated accumulation of ROS and would, in turn, result in secondary oxidative stress, causing severe cellular damage (Beck *et al.*, 2007; Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Oliver *et al.*, 1997; Toldi *et al.*, 2009). These secondary oxidative damages include the breakdown of membranes and nucleic acids, photoinhibition and photosystem damage, lipid peroxidation, and protein denaturation through sulfyhydryl group oxidation (Fernandez & Strand, 2008; Gill & Tuteja, 2010; Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Oliver *et al.*, 1997; Toldi *et al.*, 2009).

1.4.3 Low temperature (chilling and freezing)

Chilling and freezing are varying intensities of low temperature stress that are experienced by plants, and together primarily affect biomembranes, and metabolic and protein homeostasis (Beck *et al.*, 2007; Kratsch & Wise, 2000; Schulze *et al.*, 2005b; Warren, 1998; Welti *et al.*, 2002; Yadav, 2010). Chilling is regarded as low temperatures that are above the freezing point, whereas freezing incorporates the effects of chilling and involves the transition of water into ice crystals (Beck *et al.*, 2007; Schulze *et al.*, 2005b; Warren, 1998; Welti *et al.*, 2002).

The effects of chilling is usually more pronounced in tropical and subtropical plant species e.g. *Coffea arabica* (Schulze *et al.*, 2005b) and *E. grandis* (Padayachee *et al.*, 2009). Sub-tropical and tropical species, including non-acclimated temperate species, can be seen as chilling sensitive plants, where different organs, ultra-structures, and specific metabolic pathways of individual plants have varying degrees of sensitivities (Kratsch & Wise, 2000;

Schulze *et al.*, 2005b; Warren, 1998). However, at the sub-cellular level, the amount of chilling (a product of intensity and duration) can cause metabolic imbalances and membrane-associated alterations, which can negatively impact important membrane-bound processes, such as photosynthesis and respiration (Beck *et al.*, 2007; Kratsch & Wise, 2000; Schulze *et al.*, 2005b; Yadav, 2010). Thus upon exposure to chilling, pre-existing regions of limited membrane lipid mobility (due to spanning membrane proteins) will expand, thereby increasing the viscosity of the membrane (Beck *et al.*, 2007; Kratsch & Wise, 2000; Schulze *et al.*, 2005; Welti *et al.*, 2002; Yadav, 2010). Such expansions can negatively impact the maintenance of concentration gradients and active and passive protein transport, whereby transport pumps are inactivated (Beck *et al.*, 2007; Kratsch & Wise, 2000; Schulze *et al.*, 2005; Warren, 1998; Welti *et al.*, 2002; Yadav, 2010). An example of this is the decrease of proton motive force in mitochondria due to the loss of the proton gradient that is involved in the supply of adenine triphosphate (ATP).

The extent of the impact of chilling and the region of sensitivity of the membrane is dependent on the type of plant, tissue, cells, and organelles (Kratsch & Wise, 2000; Schulze *et al.*, 2005). For example, with *Eucalyptus* species impact primarily occurs in plasma membranes and with spruce the tonoplast (Schulze *et al.*, 2005b). In addition, plant circadian rhythms are also influenced by fluctuations in temperatures, because of the sensitivity of enzyme activities to temperature (Kratsch & Wise, 2000; Schulze *et al.*, 2005b; Warren, 1998). Thus, chilling would slow and impede metabolic processes, which can result in the imbalance of homeostasis (Beck *et al.*, 2007; Kratsch & Wise, 2000; Schulze *et al.*, 2005b; Warren, 1998). Furthermore, the combination of high-light intensity and chilling on photosynthesis can result in photoinhibition, where conditions can cause the uncoupling of membrane-based processes, which then leads to the formation of ROS and the onset of secondary oxidative stress (Beck *et al.*, 2007; Fernandez & Strand, 2008; Schulze *et al.*, 2005b).

Freezing, on the other hand, incorporates the effects of chilling and importantly desiccation (Beck *et al.*, 2007; Schulze *et al.*, 2005b; Warren, 1998; Yadav, 2010). During freezing, temperatures reaching the freezing point induce expanding ice nucleation that can be of intra-, inter- and/or extracellular origin e.g. from the cell wall, xylem, stomata, microorganisms, and individual proteins (Kratsch & Wise, 2000; Schulze *et al.*, 2005b; Warren, 1998). The expanding ice would entrap cellular water which disrupts its availability, as well as causing mechanical damage by rupturing cellular structures (Beck *et al.*, 2007; Kratsch & Wise, 2000;

Nagao *et al.*, 2008; Schulze *et al.*, 2005b; Warren, 1998; Welti *et al.*, 2002). This capture of water in the form of ice removes the hydrophilic medium, and thus destabilise hydrophobic interactions (Beck *et al.*, 2007; Schulze *et al.*, 2005b; Warren, 1998; Welti *et al.*, 2002). Disruption of hydrophobic interactions results in lipid phase changes, where phospholipids originating from micelles and membrane bilayer structures aggregate into lipid droplets (Kratsch & Wise, 2000; Nagao *et al.*, 2008; Warren, 1998; Welti *et al.*, 2002). These aggregates cause deformation of cellular membranes, therefore impacting cellular membrane-associated processes (Nagao *et al.*, 2008; Warren, 1998; Welti *et al.*, 2002). Furthermore, the loss of the hydrophilic medium due to freezing can cause cytorrhysis (cell wall wrinkling), which destabilizes the structural integrity and Ψ of cells e.g. the loss of turgor pressure (Beck *et al.*, 2007; Nagao *et al.*, 2008; Warren, 1998; Welti *et al.*, 2002).

1.5 Abiotic stress responses

Despite the inherent stressful effects of dehydration, desiccation and low temperature stress, plants possess mechanisms of resistance that can ensure their ability to repair and survive (Gaspar *et al.*, 2002). These mechanisms of resistance are predominantly based on the induction of adaptive metabolism, which can impart tolerance, avoidance and acclimation towards the stress (Schulze *et al.*, 2005a; Welti *et al.*, 2002; Padayachee, 2007), and thus may also help mitigate the effects of physical drying encountered during cryopreservation (Padayachee *et al.*, 2008). The integrated processes of the adaptive metabolism include stress sensing and signalling, facilitating the regulation of gene expression and cross-talk in signal transduction pathways leading to the expression and suppression of stress-related genes, and inducing the production of protective stress-related gene products (Fujita *et al.*, 2009; Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Seki *et al.*, 2007; Shinozaki *et al.*, 2003). Furthermore, adaptive metabolism is reversible when its purpose has been served as normal conditions return, but the effects of abiotic stress and its responses can create an imprint on the vegetative plant, which can result in a 'new' state of homeostasis (Bruce *et al.*, 2007; Grime & Campbell, 1991).

However, the effectiveness of adaptive metabolism in vegetative plants is dependent on the characteristics of the individual plant. These characteristics include the species and genotype, its age and stage of development, and the organ and cell type (Moore *et al.*, 2009; Schulze *et al.*, 2005b; Toldi *et al.*, 2009; Warren, 1998). This means that resistance to stress is different between plants, since these characteristics determine how the integrated processes work,

which ultimately affects the overall success of abiotic stress resistance (Moore *et al.*, 2009; Schulze *et al.*, 2005b; Toldi *et al.*, 2009; Warren, 1998).

1.5.1 Concepts in abiotic stress responses

When addressing abiotic stress responses, it is important to define certain key concepts, related to the understanding of the topic. These concepts are tolerance, avoidance, damage, repair and acclimation, which are all important contributors to resistance (Gaspar *et al.*, 2002).

1.5.1.1 Tolerance, avoidance, damage and repair

Tolerance is acquired through the maintenance of high metabolic activities during adaptive metabolism in response to 'stress' and 'strain' (Gaspar *et al.*, 2002; Kaplan *et al.*, 2004; Padayachee, 2007). Examples of this include the acquired synthesis of late embryonic abundant proteins (LEA), responsive-to-ABA proteins and dehydrins implicated in cellular protection during water stress in *Craterostigma plantagineum*, and the rapid decline of protein synthesis in *Tortula ruralis*, associated with constitutive cellular protection against desiccation (Hoekstra *et al.*, 2001; Moore *et al.*, 2009). Cold shock (4°C), for example, also increased the metabolic synthesis of proline and sucrose in *Arabidopsis thaliana*, Columbia ecotype (Kaplan *et al.*, 2004). Avoidance, in contrast, involves the reduction of primary metabolic activities for growth and development, thus resulting in a possible dormant state, when exposed to 'strain' and 'stress' of great intensity and duration (Gaspar *et al.*, 2002). Plants during drought conditions, for example, minimize water loss and increase water uptake, thus reducing photosynthesis (Chaves *et al.*, 2003). This is physiologically achieved by acquired changes including closing the stomata during the hot and dry day, and reducing light absorbance by allowing leaves to roll (Chaves *et al.*, 2003; Schulze *et al.*, 2005a). Another example is of woody perennials, which undergo the development of dormancy for overwintering during winter months (Welling & Palva, 2006). In light of the above, plants have acquired several tolerance and avoidance responses, so that resistance can be established towards the abiotic strain and stress. However, these responses are complex as plants possess several combinations of tolerance and avoidance mechanisms (Gaspar *et al.*, 2002).

Despite tolerance and avoidance responses, the issue of immediate or delayed forms of damages incurred in plant tissues and cells cannot be avoided (Gaspar *et al.*, 2002; Kranner *et al.*, 2010). These forms of damages include somaclonal variations, mutations, neoplastic progressions during *in vitro* conditions, loss of cellular and sub-cellular membrane integrity,

and ultimately death resulting from necrosis and/or apoptosis (Gaspar *et al.*, 2002; Kranner *et al.*, 2010; Schulze *et al.*, 2005a). Repair mechanisms therefore are integral for recovery of cellular, sub-cellular and molecular structures and processes, which are vital for the overall normal function and survival of the plant (Gaspar *et al.*, 2002; Kaplan *et al.*, 2004; Schulze *et al.*, 2005a). The repair of DNA after UV irradiation, is an example of this, since the irradiation can damage leaves and trigger senescence (Chaves *et al.*, 2003; Schulze *et al.*, 2005a). Although there is the ability to repair, it is important that plants limit stress-related damage to recoverable levels (Oliver *et al.*, 1997 and 1998). Reviews by Oliver *et al.* (1997 and 1998) have suggested that fully desiccation-tolerant plants limit damage to repairable levels, which was achieved by the means of tolerance and avoidance.

1.5.1.2 Acclimation

In the natural environment, abiotic conditions continually fluctuate on a daily and seasonal temporal scale (Schulze *et al.*, 2005b). These continuous fluctuations and changes constantly make plants adjust their cellular state to further accommodate the environmental conditions ahead, which causes subsequent adjustments in the molecular, metabolic, biochemical and physiological attributes (Gaspar *et al.*, 2002; Grime & Campbell, 1991; Levitt, 1980b; Nilsen & Orcutt, 1996). These constant adjustments at the cellular level are referred to as cellular acclimation, and are associated with the improvement of the overall tolerance of the plant towards abiotic stress (Gaspar *et al.*, 2002; Grime & Campbell, 1991; Levitt, 1980b; Nilsen & Orcutt, 1996).

Acclimation, or hardening in the case of the whole plant, is especially well documented with low temperature stress (Levitt, 1980b), both in the natural environment and *ex situ* conditions (see mini-reviews and opinions: Stitt & Hurry, 2002; Welling & Palva, 2006; Wisnieski *et al.*, 2003). This acclimation is commonly known as cold acclimation. In brief, cold acclimation involves the prolonged exposure of low temperature-tolerant plants to chilling conditions, which causes adjustments in growth and metabolism, and with this subsequently results in the improvement of freezing tolerance in the plant (Gaspar *et al.*, 2002; Nilsen & Orcutt, 1996).

1.5.2 Adaptive metabolism: biochemical responses

Tolerance, avoidance, repair and acclimation rely on the activities of plant adaptive metabolism, which is triggered by upstream molecular processes that result in changes with biochemical responses from its ‘normal’ state (Gaspar *et al.*, 2002; Kaplan *et al.*, 2004;

Shinozaki *et al.*, 2003). These responses trigger and induce metabolic diversions and re-allocations of primary metabolites and biomolecules, such as stored starch, other carbohydrates, lipids, amino acids and proteins already functioning during primary metabolism of growth and development (Gaspar *et al.*, 2002; Kaplan *et al.*, 2004). The diversions and re-allocations result in the synthesis of primary (carbohydrates and proline – the specific biomolecules of interest in the present study) and secondary metabolites (phenolic acid) for protective, signalling and regulatory purposes (Kaplan *et al.*, 2004; Shinozaki *et al.*, 2003). Subsequently, this adaptive metabolism is involved in the downstream physical and physiological responses towards the abiotic stress.

1.5.2.1 Carbohydrates (soluble sugars)

Alteration of carbohydrate metabolism has been identified as one of the strategic responses elicited during the onset of dehydration, desiccation and low temperature stress (chilling and freezing) (Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Schulze *et al.*, 2005; Seki *et al.*, 2007). Accumulated soluble sugars (such as sucrose) and non-reducing raffinose-family oligosaccharides (RFOs) result in osmotic adjustments, whereby the metabolically favourable Ψ gradient is maintained and cellular turgor is protected (Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Seki *et al.*, 2007). As such, these metabolites assume the role of osmoprotectants. At the same time, soluble sugars act as water-replacing surrogates in cells that require hydrogen bonds for molecular assembly and maintenance (Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Schulze *et al.*, 2005; Seki *et al.*, 2007). This therefore ensures controlled structural stabilization of macromolecules by surrogate-mediated preferential hydration, which may allow sensitive biomolecules, such as enzymes, to remain stable under a dried state (Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Schulze *et al.*, 2005; Seki *et al.*, 2007).

This surrogate role is even more important in the preservation of the lipid bilayer of biomembranes (Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Schulze *et al.*, 2005; Seki *et al.*, 2007). For example, RFOs have been specifically implicated in substituting water molecules in the hydration films of lipid head groups therefore preventing deleterious lipid phase transitions during freezing (Zuther *et al.*, 2004). The high concentration of these soluble sugars could vitrify the cytoplasm, thereby preventing the crystallization of embedded chemical compounds, fusion of membrane systems and conformational changes in proteins (Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Seki *et al.*, 2007). Furthermore, these soluble sugars are believed to lower the freezing point during freeze-desiccation, hence further

lowering the temperatures necessary for ice nucleation (Schulze *et al.*, 2005). Thus, soluble sugars are also considered as cryoprotectants (Engelmann, 2004; Gonzalez-Arnao *et al.*, 2008).

1.5.2.2 Phenolic acid

Phenylpropanoids (phenolic acid) are well documented protective secondary metabolites that are produced and accumulated when plants experience abiotic stress e.g. dehydration and low temperature (Chung *et al.*, 2006; Dixon & Paiva, 1995; Kirakosyan *et al.*, 2004; Padayachee *et al.*, 2008; Pennycooke *et al.*, 2005; Solecka, 1997). These secondary metabolites are produced from phenylalanine, where it is converted into cinnamate by L-phenylalanine ammonia-lyase (Dixon & Paiva, 1995; Solecka, 1997). Cinnamate is the backbone for the synthesis of the two distinct families of phenolic acid, namely those derived from benzoic acid (e.g. gallic acid and salicylic acid) and cinnamic acid, where the latter family gives rise to the synthesis of lignin, flavanoids and anthocyanins (Dixon & Paiva, 1995; Steyn *et al.*, 2002; Weidner *et al.*, 2000; Solecka, 1997). Phenolic acids rarely occur in their free form but instead are usually associated with conjugant sugars, organic acids and cell wall carbohydrates (Dixon & Paiva, 1995; Kirakosyan *et al.*, 2004; Pennycooke *et al.*, 2005; Solecka, 1997; Weidner *et al.*, 2000). These biomolecules are well known for their antioxidant activity by quenching free-radicals and stress-related ROS, thus preventing subsequent oxidative damage of cells (Dixon & Paiva, 1995; Padayachee *et al.*, 2008; Pennycooke *et al.*, 2005; Solecka, 1997; Weidner *et al.*, 2000).

Phenolic acid have also been shown to be involved in improving the affinity between cell membranes and walls, hence preventing membrane collapse when cells are experiencing conditions of extreme water loss (Pennycooke *et al.*, 2005; Solecka, 1997). To be more specific, complexes of these biomolecules result in the formation of lignin and suberin that are incorporated into cell wall structures, which ensures that the water status of tissues are maintained, since both act as water barriers (Dixon & Paiva, 1995; Pennycooke *et al.*, 2005; Solecka, 1997). In addition, anthocyanins and flavonoids derived from cinnamic acid are involved in the attenuation of photoinhibition and photoxidative damage by controlling the amount of visible light and UV-irradiation reaching photosynthetic cells, whenever metabolic activities are stressed e.g. by chilling or dehydration (Close *et al.*, 2000; Dixon & Paiva, 1995; Solecka, 1997; Steyn *et al.*, 2002).

1.5.2.3 Proline

The accumulation of the amino acid proline is another strategic response to abiotic stresses, which, like soluble carbohydrates, also assumes the role as an osmoprotectant (Ashraf & Foolad, 2007; Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Schulze *et al.*, 2005a; Seki *et al.*, 2007). Proline is an amphiphilic osmolyte that has a hydrophobic moiety that is able to bind to hydrophobic surfaces and convert them to hydrophilic; consequently it can act as a water replacement molecule and provide a 'hydration effect' (Stein *et al.*, 2011; Tantau *et al.*, 2004). It can be produced either from glutamate or ornithine, and is accumulated in the cytosol but there is also recent evidence of its accumulation within chloroplasts (Stein *et al.*, 2011; Tantau *et al.*, 2004). The roles of proline include functioning as a stabilizer of sub-cellular structures (e.g. proteins), scavenger of free radicals and ROS (antioxidant role), and acting as a cellular reduction-oxidation (REDOX) potential buffer during respiration and photosynthesis (Ashraf & Foolad, 2007; Hoekstra *et al.*, 2001; Moore *et al.*, 2009; Reierson & Rees, 2001; Schulze *et al.*, 2005a; Seki *et al.*, 2007). It is also considered a protein-compatible hydrotope that protects complex quaternary protein structures, and is also involved in maintaining the NADP/NADPH ratios for metabolism (Ashraf & Foolad, 2007; Stein *et al.*, 2011). In addition, proline can be catabolised to provide relief for stress recovery in the form of reducing agents (carbon and nitrogen) in supporting mitochondrial oxidative phosphorylation and ATP generation for stress recovery and repair, along with the amino acid itself being a building block for protein synthesis (Ashraf & Foolad, 2007; Jouve *et al.*, 1993).

1.5.2.4 Reactive oxygen species (ROS)

Alterations in the REDOX potential is a common response when plant cells and tissues are exposed to abiotic stresses, such as dehydration, desiccation and low temperature (Bowler & Fluhr, 2000; Foyer & Noctor, 2005). This is caused by alterations in the levels of ROS such as superoxide, singlet oxygen, hydrogen peroxide, and hydroxyl radicals (Bowler & Fluhr, 2000; Foyer & Noctor, 2005; Gaspar *et al.*, 2002). The generation of ROS is usually incurred from disequilibria in electron transfer reactions of organelles (e.g. chloroplasts and mitochondria), which results in the production of superoxide within the organelles and then the cells (Bowler & Fluhr, 2000; Fernandez & Strand, 2008; Foyer & Noctor, 2005; Gaspar *et al.*, 2002). Superoxide can be further reduced to produce other ROS, such as hydrogen peroxide and hydroxyl radicals (Gaspar *et al.*, 2002). Collectively, ROS cause severe cellular damage that can lead to lipid peroxidation (oxidation of lipids into free fatty acids),

photosynthetic pigment loss and photosystem damage, DNA mutations, and protein denaturation via sulfhydryl group oxidation (Bowler & Fluhr, 2000; Fernandez & Strand, 2008; Gaspar *et al.*, 2002; Moore *et al.*, 2009; Schulze *et al.*, 2005).

Although more commonly associated with oxidative stress, ROS has also gained much attention as a role player in stress-related signalling (Bowler & Fluhr, 2000; Foyer & Noctor, 2005). It has been suggested that plant cells are genetically programmed to produce ROS which is induced throughout the course of development and environmental fluctuations, and could subsequently cause complex downstream effects on primary and secondary metabolism (Foyer & Noctor, 2005; Fujita *et al.*, 2009). In particular, superoxide and hydrogen peroxide have been considered as secondary messengers involved in many processes associated with growth and development, as well as implicated in necrosis and apoptosis (Foyer & Noctor, 2005; Fujita *et al.*, 2009; Gaspar *et al.*, 2002). Furthermore, superoxide bursts at the plasma membrane level have been identified as a major way of transmitting information about external environmental fluctuations into plant cells (Foyer & Noctor, 2005).

1.5.3 Cross-talk: 'cross-acclimation' and 'cross-tolerance'

There has been an increased understanding about plant responses and tolerance to individual abiotic stresses (dehydration, desiccation, chilling, and freezing), however, in many instances abiotic and even biotic stresses occur in combinations (Mittler, 2006). Thus, it has been suggested that there may be similar response and tolerance mechanisms in place which allow plants to tolerate combinations of stresses (Mittler, 2006). This has led to the hypothesis of cross-talk (Beck *et al.*, 2007; Fujita *et al.*, 2009; Mittler, 2006; Rizhsky *et al.*, 2004; Seki *et al.*, 2002; Shinozaki *et al.*, 2003; Shinozaki & Yamaguchi-Shinozaki, 2000), and is supported by evidence at the molecular level. For example, a variety of genes are induced by both low temperature and dehydration, in addition to individual stress-specific inductions (Beck *et al.*, 2007; Shinozaki *et al.*, 2003; Shinozaki & Yamaguchi-Shinozaki, 2000). This is suggestive of similar biochemical processes functioning in both dehydration- and low temperature-stress responses, and this may involve the regulation of genes implicated in transducing the stress response signal, and production of protective metabolites for tolerance (Shinozaki *et al.*, 2003; Shinozaki & Yamaguchi-Shinozaki, 2000).

Most support of cross-talk has been documented primarily at the molecular and transcriptional level (Hirayama & Shinozaki, 2010). Reports have shown that many *cis*- and *trans*-acting elements are key contributors, and these may be controlled by both abscisic acid

(ABA)-dependent and -independent regulatory systems (Fujita *et al.*, 2009; Mittler, 2006; Shinozaki *et al.*, 2003; Shinozaki & Yamaguchi-Shinozaki, 2000). Together they have been identified in stress-responsive transcription, therefore involved in cross-talk between dehydration- and low temperature-stress signalling pathways, which integrates different networks including mitogen-activated protein kinase (MAPK) cascades (Beck *et al.*, 2007; Fujita *et al.*, 2009; Mittler, 2006; Shinozaki *et al.*, 2003; Shinozaki & Yamaguchi-Shinozaki, 2000).

Even though there have been improvements in our knowledge on cross-talk, at present there is a growing necessity to understand the downstream effects of cross-talk at a biochemical and physiological perspective in plants (Beck *et al.*, 2007; Chinnusamy *et al.*, 2004; Hirayama & Shinozaki, 2010; Mittler, 2006). More important is the question of how the molecular process of cross-talk translates into its natural tolerance to combinations of stresses, since molecular responses are closely linked between different stresses (Chinnusamy *et al.*, 2004; Hirayama & Shinozaki, 2010; Mittler, 2006). In other words, the question of whether acclimation under one abiotic stress (i.e. chilling) induce natural resistance to another completely different abiotic stress (i.e. dehydration) has been raised. More specifically, can 'cross-acclimation' result in 'cross-tolerance'?

Thus, it is important to understand the downstream biochemical and physiological responses of cross-talk and whether 'cross-acclimation' would promote 'cross-tolerance' based on the analyses of underlying physiological and biochemical responses. This understanding would contribute in developing plants more tolerable to combinations of abiotic stresses, by relying on the flexibility of the natural resistance mechanisms in plants. For the purpose of this study, 'cross-acclimation' and 'cross-tolerance' was viewed as a possible method of improving tolerance and ensuring viability of *in vitro E. grandis* materials that require pre-treatment by physical drying in order to be successfully cryopreserved.

1.6 Aims of the study

Cryopreservation pre-treatment of isolated *in vitro E. grandis* materials by physical drying reduces the viability of the samples even before the later stages of the process (Padayachee *et al.*, 2008). For conservation purposes, it is therefore important to ensure that viability is retained after the cryopreparative drying during pre-treatment. Abiotic stresses are commonly experienced in the natural environment, and *E. grandis*, like all plants, may possess the

natural resistance mechanisms to trigger and induce tolerance and acclimation towards these artificial abiotic stresses.

Thus, the aims of the study were to determine whether pre-exposure of *in vitro E. grandis* shoots to the low temperature stress of chilling could induce 'cross-tolerance' to water loss during cryopreparative drying (physical drying), and to find evidence for the downstream effects of 'cross-tolerance'. This was achieved by understanding the biochemical and physiological responses of *in vitro E. grandis* shoots towards chilling and physical drying, on an individual basis and in combination.

The study investigated these aims through four progressive stages. Firstly, it was necessary to establish a fundamental understanding of the biochemical and physiological responses of *E. grandis* to the *in vitro* conditions. Secondly, the biochemical and physiological responses of *in vitro E. grandis* materials to low temperature stress of chilling had to be determined in order to select the appropriate chilling temperature and culture period for acclimation. Thirdly, the fundamental biochemical and physiological responses of the *in vitro E. grandis* material to a suitable physical drying period had to be established. The final stage involved the pre-exposure of the *in vitro E. grandis* materials to the selected chilling temperature and exposure period, followed by physical drying. The biochemical and physiological responses were determined by measuring changes in water content, viability and vigout, and changes in selected stress-associated biomolecules and metabolites (total soluble sugars, starch, phenolic acid, proline and superoxide).

Through these successive investigations, the study provided answers to the following questions specifically posed with regard to the aims of study:

- What is the appropriate pre-exposure chilling temperature to influence the status of stress-associated biomolecules and metabolites with the potential to improve tolerance to cryopreparative drying?
- What is the appropriate pre-exposure period to the chilling temperature necessary to influence the status of potentially protective stress-associated biomolecules and metabolites?
- Does the formation of protective stress-associated biomolecules and metabolites influenced by chilling cause greater tolerance of *in vitro E. grandis* shoots to

cryopreparative drying (i.e. does chilling induce cross-tolerance to cryopreparative drying)

- How do the stress-associated biomolecules and metabolites relate to cross-tolerance?
- How do the stress-associated biomolecules and metabolites synergistically relate to cross-tolerance?
- Can cross-tolerance be induced in *E. grandis in vitro* shoots?

1.7 Structure of dissertation

This dissertation is presented in such a way that the materials and methods are a stand-alone chapter, and consequently that section is excluded from Chapters 3-6 (experiments). In other words, details included in the material and method is not repeated in subsequent chapters. This is because the materials and methods (i.e. physiological and biochemical measurements, microscopy, and statistical analysis) were commonly applied to all the experiments addressing the objectives, where each experiment (chapter) was specific to the abiotic stress treatment.

CHAPTER 2. MATERIALS AND METHODS

2.1 Material

Eucalyptus grandis, sponsored by Mondi Group SA, was used in the research. Rooted-cuttings of *E. grandis* were maintained in the greenhouse at the University of the Witwatersrand, Johannesburg (Fig 2). The plants were watered at 8 hour (h) intervals for 15 minutes (min), and were sprayed with the following fertilisers – 10 $g.l^{-1}$ Mondi Orange (Mondi SA), 0.3 $g.l^{-1}$ Calmag and 3.33 $ml.l^{-1}$ Nitrosol® once per week at a weekly rotation. For the first 6 weeks, the foliar fungicide Bravo 720® (2 $ml.l^{-1}$) and soil fungicide Folicular/Sporgon were each sprayed once per alternating week. After the first 6 weeks, the soil fungicide was changed to Previcure (1 $ml.l^{-1}$) and this was applied only once a month while Bravo 720® was sprayed every second week. The Previcure and Bravo 720® fungicide treatment cycle and the weekly fertilizer treatment were continued throughout the project.



Fig 2. Greenhouse maintained *Eucalyptus grandis* at the University of the Witwatersrand, Johannesburg that were originally established from rooted cuttings sponsored by Mondi Group SA.

2.2 Establishing *in vitro* material

In vitro E. grandis shoot clusters (shoots) were established from greenhouse materials once a month, thereby generating a continuous supply of experimental materials. *In vitro* materials used for experiments were randomly selected from established *in vitro* cultures aged 5-11 months. Materials older than 11 months were discarded and replaced with newer cultures in order to maintain the age class of the *in vitro* materials used. Such ages of materials were selected because this study involved extended experiments that required a longer time schedule to accomplish. Furthermore, materials that are serially sub-cultured *in vitro* for a longer period can undergo neoplastic progression that can lead to the progressive loss of organogenic totipotency (Gaspar *et al.*, 2000; Harding *et al.*, 2009).

2.2.1 Surface decontamination

Under sterile conditions, nodal explants obtained from greenhouse-maintained *E. grandis* were initially surface-decontaminated with 0.2 $g.l^{-1}$ mercuric chloride for 5 min followed by thorough rinsing with sterile ultra-pure water (MilliQ®). The explants were further decontaminated with 2% (w/v) sodium hypochlorite (commercial bleach) for 5 min and then thoroughly rinsed. The explants were then immersed for final decontamination in a 0.01 $mg.ml^{-1}$ streptomycin and 0.025 $\mu g.ml^{-1}$ amphotericin solution (Sigma Antibiotic and Antimycotic Solution) for 20-30 min (Padayachee, 2008; Watt *et al.*, 1996).

2.2.2 *In vitro* development

Following surface-decontamination, the nodal explants were trimmed and then cultured onto 50mL of semi-solid multiplication medium in magenta jars. The components of the multiplication medium were 4.42 $g.l^{-1}$ (full strength – Highveld Biological®, South Africa) Murashige and Skoog (MS; 1962) culture medium that was supplemented with 25 $g.l^{-1}$ sucrose, 3 $g.l^{-1}$ Gelrite®, 0.2 $mg.ml^{-1}$ 6-benzylaminopurine (BAP), 0.01 $mg.ml^{-1}$ 1-naphthaleneacetic acid (NAA), and 0.1 $mg.ml^{-1}$ biotin and calcium pentothenate at pH 5.6-5.8 (Padayachee, 2007). Only visible buds and shoots from the explants were excised after 5-7 days and transferred onto fresh multiplication medium. Thereafter, the newly established *in vitro* materials i.e. stock cultures were sub-cultured every 8 weeks onto fresh multiplication medium.

When the stock cultures were required for experimental purposes, established shoots were pre-cultured onto acclimation medium for three days before being used i.e. acclimated for 3

days. The acclimation medium consisted of 4.42 $g.l^{-1}$ MS culture medium supplemented with 25$g.l^{-1}$ sucrose and 3$g.l^{-1}$ Gelrite® (Padayachee, 2007).

All *E. grandis in vitro* cultures were maintained under standard growth conditions, in a growth room maintained at 25 ± 3°C with a 14 h photoperiod (100 µmol/m²/s¹ photosynthetic photon flux density) and 10h dark period (Padayachee, 2007).

2.3 Experimental procedures

The experimental process is summarised in Fig 4. Experimental materials used were either *E. grandis* shoot clusters (i.e. shoots, Fig 3) or ground shoots (i.e. ground with liquid nitrogen, LN). After each treatment, biochemical measurements viz. total soluble sugars (TSS), starch, phenolic acid, superoxide, and proline, as well as physiological measurements viz. water content (WC), and viability and vigour were evaluated. Shoots were directly used in the evaluation of WC, viability and vigour, and superoxide assays while ground shoots were used in phenolic acid, proline, starch and TSS assays. Throughout the research, three replicates were conducted per experimental measurement, and from each replicate 3 sub-samples were assayed for TSS, proline, starch, superoxide and phenolic acid.

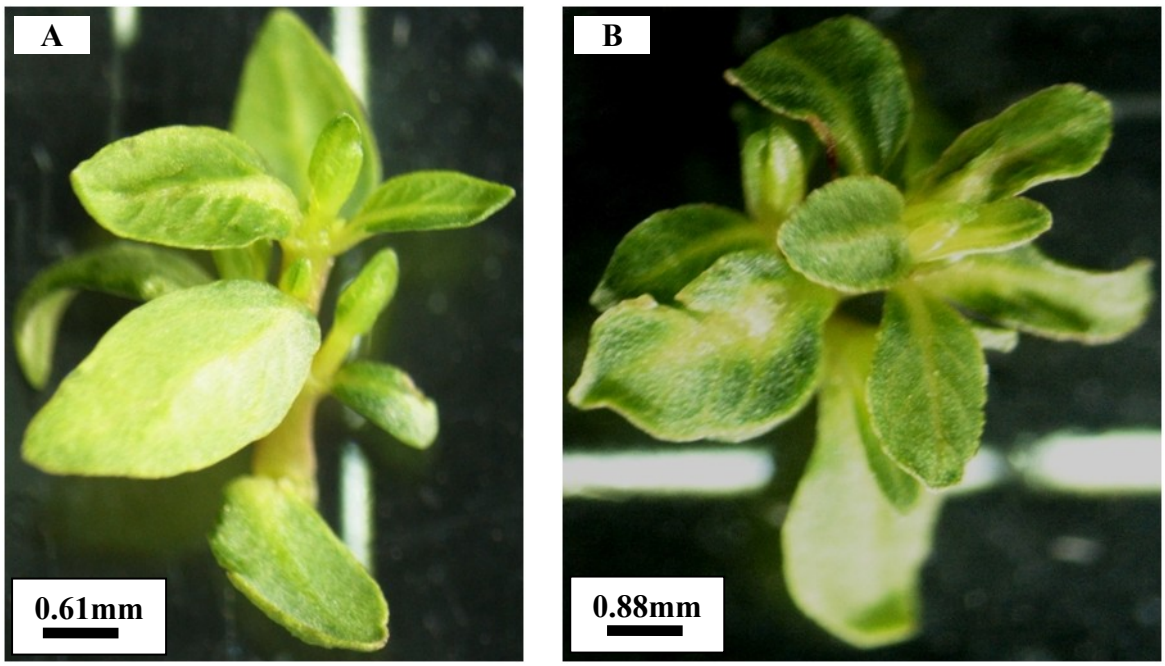


Fig 3. Longitudinal (A) and top (B) representation of shoot clusters (shoots). A shoot cluster was characterised by 4-8 developing and developed leaves and 2-5 axillary buds and was approximately 0.52 ± 0.11 cm in length and 0.69 ± 0.10 cm in width.

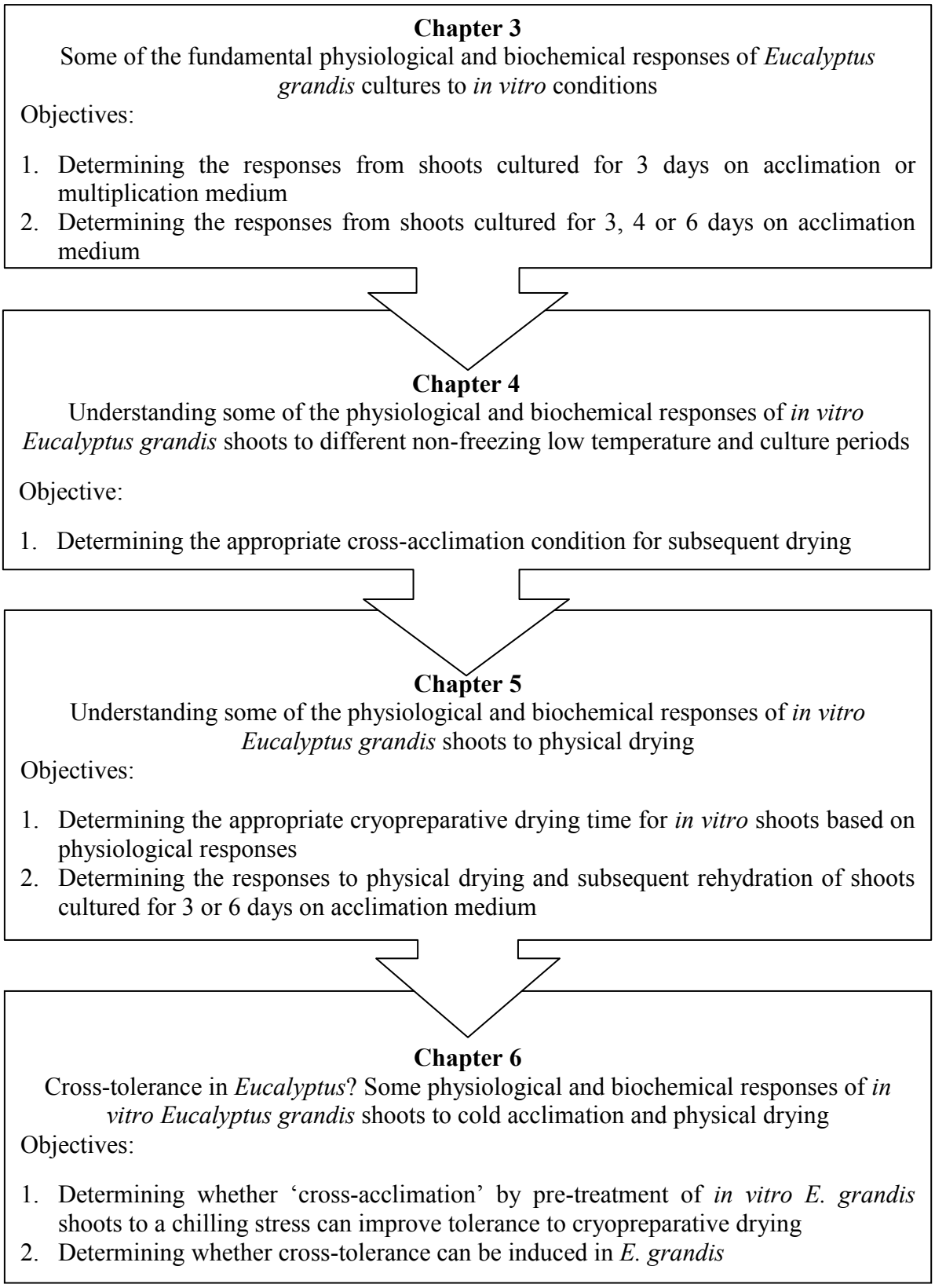


Fig 4. An overview of the experimental process. Each stage provides the title as described in Chapter 3, 4, 5 and 6, and their associated objectives

2.3.1 Biochemical and physiological responses of *in vitro* cultures

Shoots were isolated from the stock cultures and cultured onto acclimation or multiplication medium for 3 days (Fig 5). After the 3 days, biochemical and physiological responses were determined (Fig 5). This established responses under *in vitro* conditions and between the different media (Fig 5). The aforementioned, biochemical and physiological responses were also established for shoots cultured for 3, 4 or 6 days on acclimation medium (Fig 5).

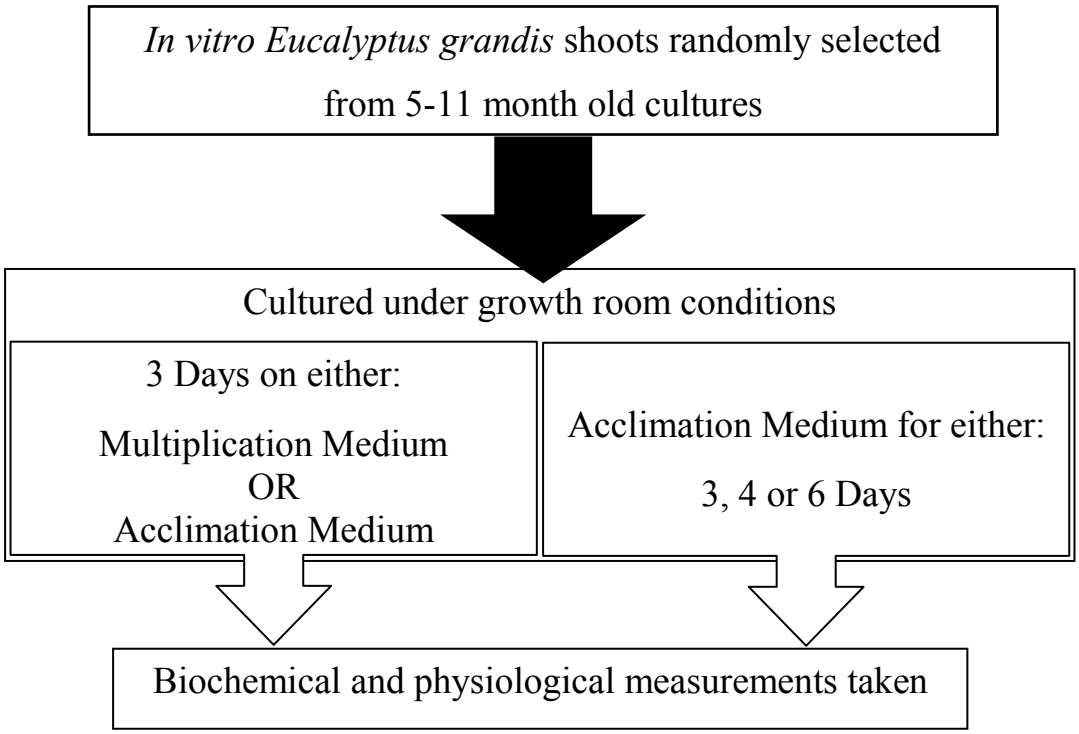


Fig 5. Outline of the determination of biochemical and physiological responses from *in vitro E. grandis* cultures on acclimation or multiplication medium, and for shoots cultured on acclimation medium for either 3, 4 or 6 days

2.3.2 Determining the appropriate cryopreparative drying time (physical drying) for *in vitro E. grandis* shoots

Randomly selected shoots (n=15) were exposed to the following drying times: 0 (control), 20, 40, 60 and 80 min (Fig 6). The shoots were dried over 400 g of activated silica gel in a 4500cm^3 desiccator at room temperature under sterile conditions with minimal illumination (described in Section 2.3.5). The WC was recorded immediately after drying (detailed in Section 2.4.1), and viability and vigour was evaluated over a period of 2 weeks (detailed in Section 2.4.2). The appropriate cryopreparative drying time (physical drying) was selected by considering the water content and physiological results as well as by statistical evaluation (Section 2.6) of the different drying times (Fig 6).

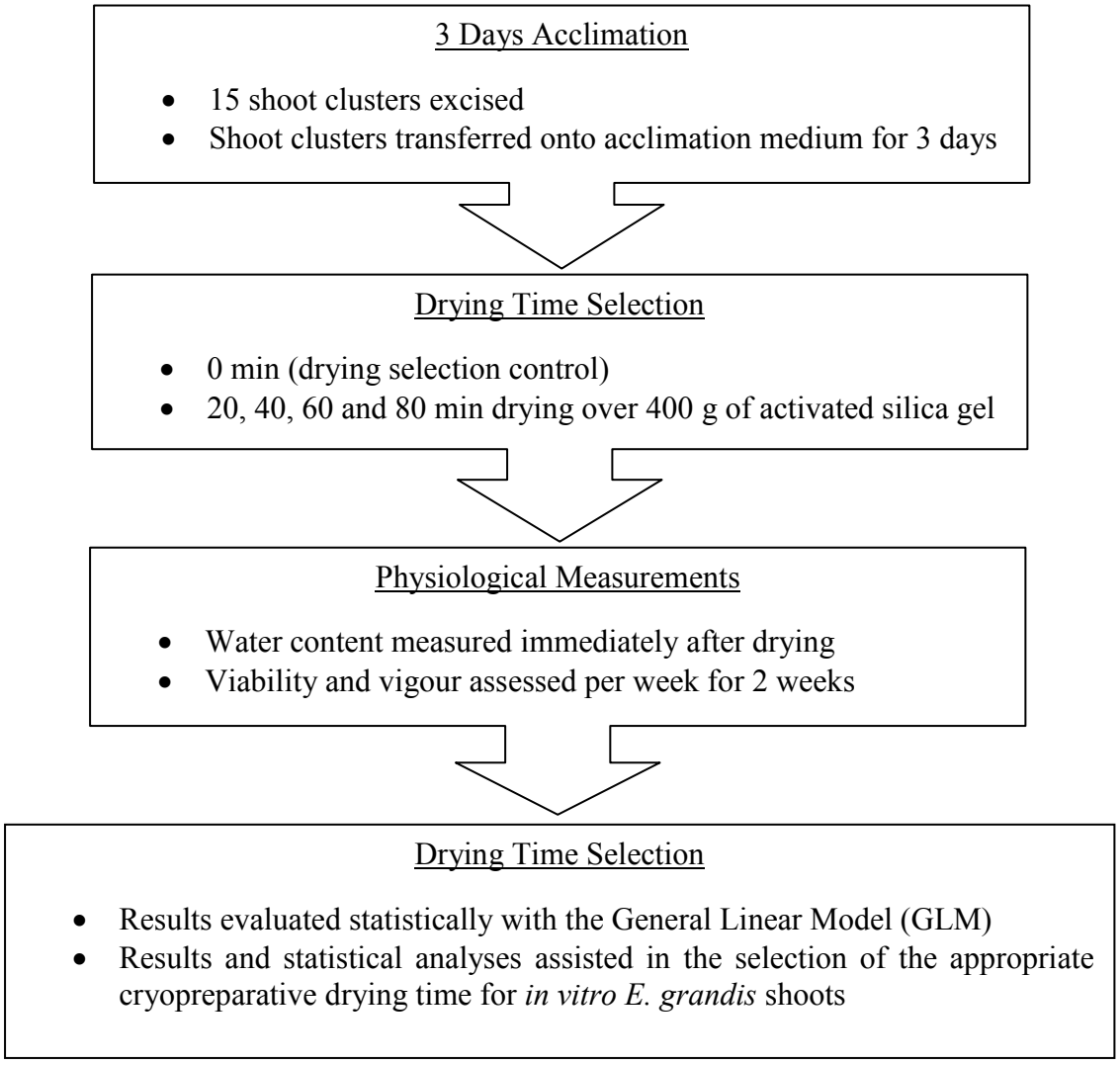


Fig 6. Summarised selection process for the appropriate cryopreparative drying time for *in vitro E. grandis* shoots. The bullet points represent the process in which the cryopreparative drying time selection was conducted

2.3.3 Controls

Controls were conducted for the 3 main experimental factors under consideration: 1) non-freezing low temperature (chilling), 2) appropriate exposure period, and 3) appropriate physical drying period. The controls were conducted in parallel to the experiments.

2.3.3.1 Controls for the chilling and exposure period experiments

These controls were the standard 3 days of growth on acclimation medium followed by continued maintenance on acclimation medium for an additional 1 day (Control A) or 3 days (Control B) under growth room conditions (Fig 7). Biochemical and physiological measurements were determined after these periods (i.e. after 4 days - Control A, or after 6 days - Control B, on acclimation medium; Fig 7).

2.3.3.2 Controls for cryopreparative drying (physical drying) experimental treatments

This control (Control C) involved the exposure of either Control A or B (described above) to the pre-determined physical drying time (Section 2.3.2). The selection of Control A or B depended on which chilling temperature and exposure period was selected (Fig 7), based on the outcome of the results of the investigation (Section 2.3.4).

For Control C, WC, proline, starch and TSS were assayed at 0 and 24 h after drying. Phenolic acid and superoxide was assayed at 0, 12 and 24 h after drying (Fig 7). The 12 and 24 h evaluation were assayed after the shoots were dried and subsequently transferred to acclimation medium and returned to growth room conditions (Fig 7).

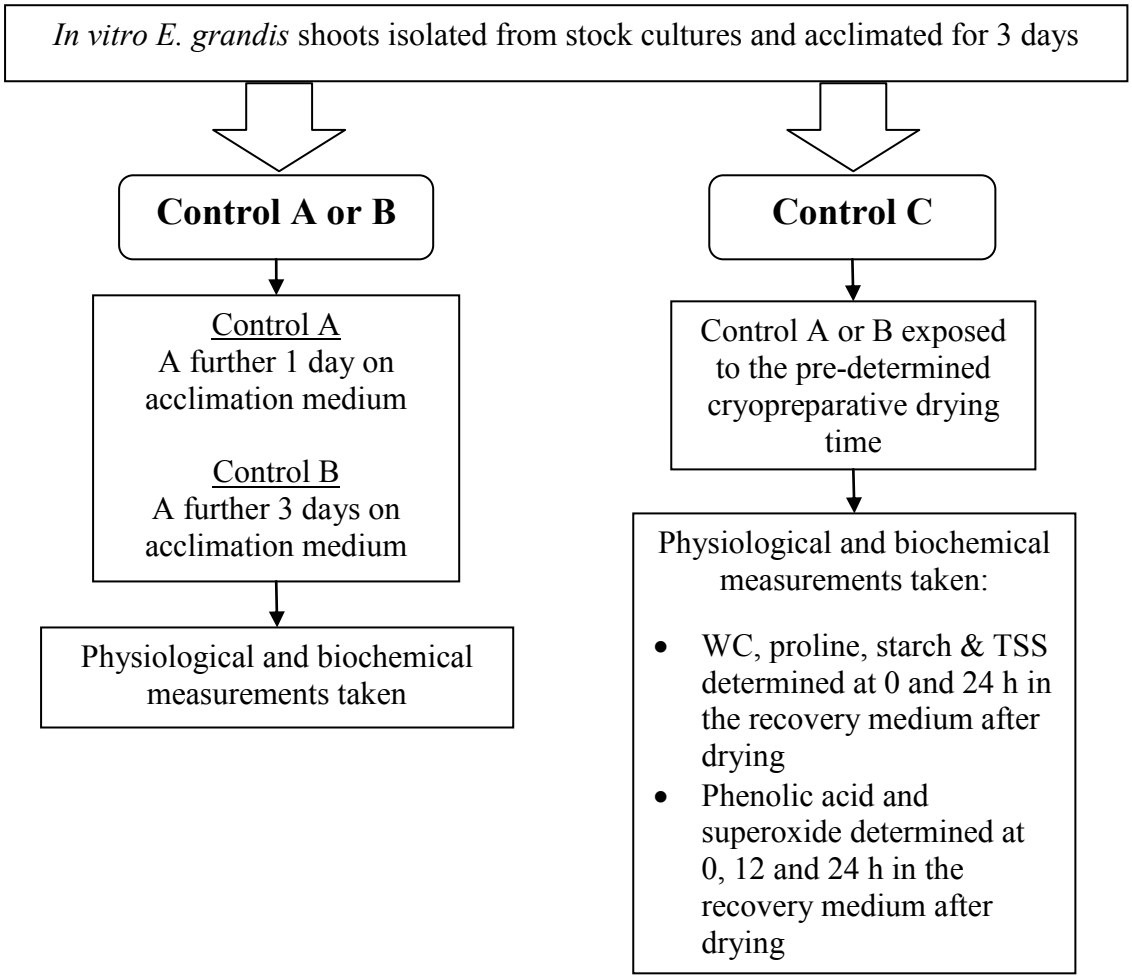


Fig 7. Summarised outline of the experimental treatment controls. Chilling and exposure controls: 1 day (Control A) and 3 days (Control B) exposure controls. Cryopreparative drying (physical drying) control: Control C; WC: water content; and TSS: total soluble sugars

2.3.4 Determination of the chilling temperature and exposure period (cold acclimation condition) for cross-acclimation

Shoots were exposed to the chilling temperatures of 5.0 ± 1.0°C, 10.0 ± 1.0°C and 15. 0± 1.0°C (Labcon Growth Chamber, South Africa) with a 14 h photoperiod and 10 h dark period (Padayachee, 2007). With each temperature, the duration of exposure was both 1 and 3 days (Fig 8). All biochemical and physiological measurements were evaluated immediately after 1 and 3 days exposure to the specified temperature (Fig 8). Based on the responses, the appropriate chilling temperature and exposure period was selected as a pre-treatment regime preceding cryopreparative drying treatment (Fig 8).

2.3.5 Cryopreparative drying (physical drying) experimental treatments

For Control C (Section 2.3.3.2) and the selected chilling and exposure pre-treatment (cold acclimation), the shoots were transferred to drying conditions (Fig 8) as described by the cryopreservation drying protocol of Padayachee (2008). Shoots were transferred onto aluminium foil sheets (65×70 mm) and were left open on the foil, and placed over 400 g activated silica gel in a 4500 cm^3 desiccator at room temperature under sterile conditions for a selected drying time (Section 2.3.2). The silica gel was activated by prior incubation at 80°C for 24 h before being used for experiments and then cooled to room temperature.

After drying, biochemical and physiological measurements were taken (Fig 8). Phenolic acid and superoxide concentration was measured over 0, 12 and 24 h, while WC, proline, starch and TSS were determined after 0 and 24 h of drying (Fig 8). The 12 and 24 h evaluations were measured after the shoots were transferred onto acclimation medium and returned to growth room conditions.

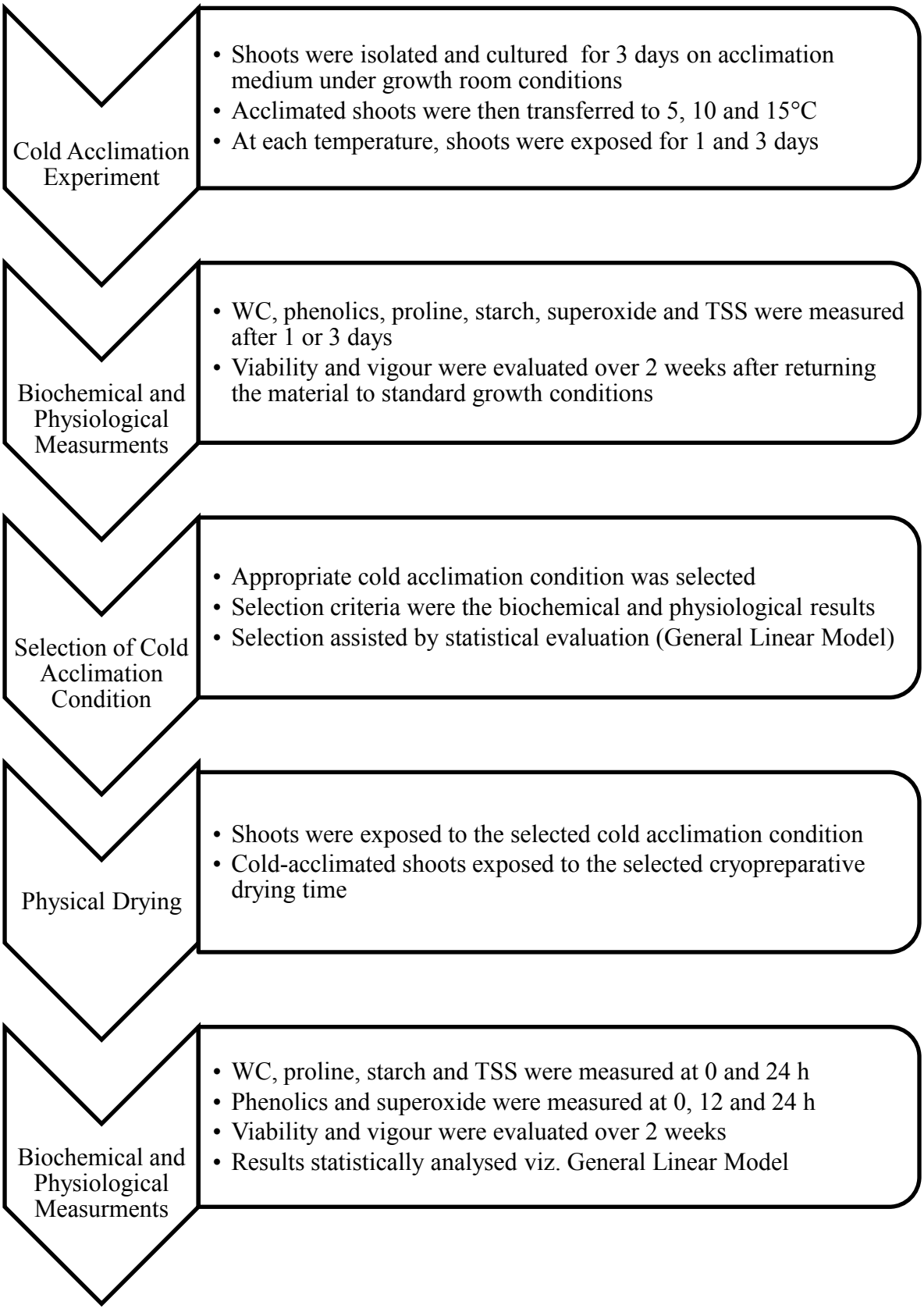


Fig 8. Summarised process of cold acclimation and cryopreparative drying experimental treatments. The bullet points indicate the process in which the experiment was conducted. WC: water content; and TSS: total soluble sugars.

2.4 Physiological measurements

The physiological measurements considered were water content (WC), viability and vigour. The WC was measured immediately after both the chilling temperature and exposure duration treatments (Section 2.3.4), while WC following physical drying were measured immediately (0 h) after drying and 24 h after reimbibition on medium (Section 2.3.5). Vigour and viability were evaluated after treated shoots were transferred onto multiplication medium, and maintained for 2 weeks under standard growth conditions.

2.4.1 Water content (WC)

Water content was determined gravimetrically. The fresh mass of 15 shoot clusters (shoots) was determined (Precisa 125A SCS, United Kingdom). The shoots were then dried in the oven for 24 h at 80°C. Post-drying, the dry mass was determined. The difference between the fresh and dry mass of shoots represented the amount of water in the shoots (Padayachee *et al.*, 2008). Water content was expressed as grams of water per gram of fresh weight of shoots (FWS).

2.4.2 Viability and vigour

Fifteen randomly selected shoots were cultured individually in culture tubes containing 10mL of multiplication medium. Viability was determined based on the percentage (%) survival of the shoots immediately after the experimental treatments (i.e. week 0) and after 2 weeks. Vigour was evaluated based on the following factors: number of visible axillary buds, number of visible shoots, callus formation (score between 0-5) (Table 2) and tissue browning (score between 0-5) (Table 3). The number of visible axillary buds, number of visible shoots, and callus formation was evaluated for only viable shoots while tissue browning was evaluated for all 15 shoots.

An axillary bud was characterised by a bud that had no leaves or an extended bud that had less than 4 small developing leaves with no indication of new buds forming at the leaf-node junction (Fig 9). A shoot was characterised by having more than 4 developing and developed leaves and/or had more than 1 visible axillary bud (Fig 9). These factors were evaluated immediately after the treatment and then after 2 weeks. Callus formation and tissue browning scores were evaluated per week over 2 weeks.

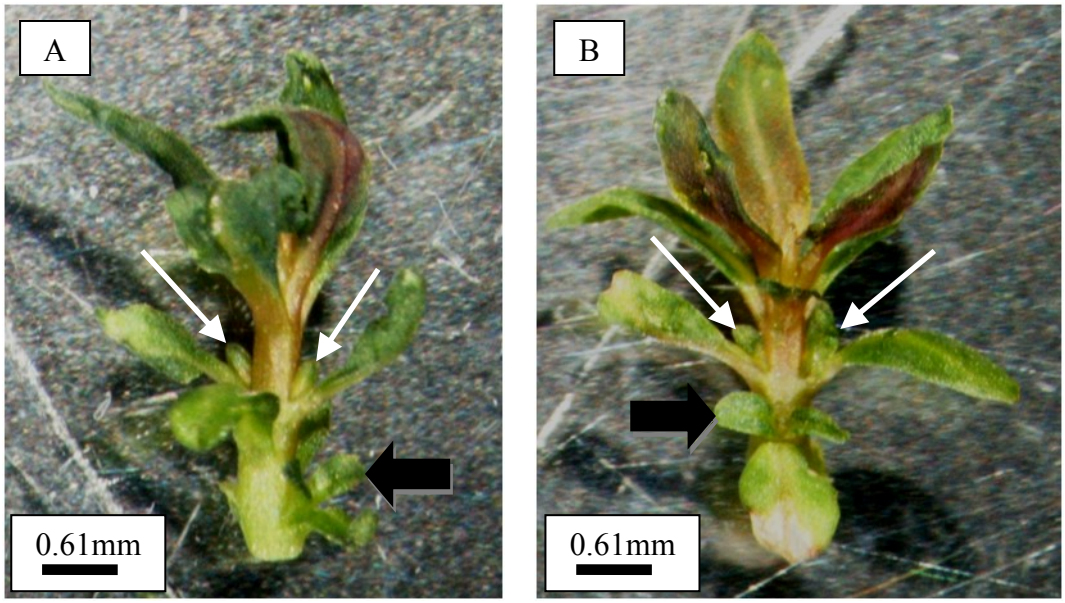


Fig 9. An *in vitro E. grandis* shoot cluster (A and B) depicting some examples of visible shoots and axillary buds identified during vigour evaluations. White arrow: (A) axillary buds, and (B) axillary buds that have developing leaves. Black arrow: (A and B) a shoot showing 2 developed and 2 developing leaves (developing leaves are not as clear).

Table 2. Score criteria for the evaluation of callus formation

Score	Criteria for score	Visual example of score criteria
0	No visible signs of callus	
1	Isolated signs of callus on leaves, stem or axillary buds (indicated by black arrow)	
2	Visible signs of callus on leaves and/or stem and/or axillary buds (indicate by black arrows)	
3	Increased signs of callus spread throughout leaves and/or stem and/or axillary buds (indicated by black arrows)	
4	Shoot cluster has been covered with callus which resulted in growth deformities	Not Available
5	Shoot cluster has been dominated by callus in its entirety resulting in impeded growth	Not Available

Table 3. Score criteria for the evaluation of tissue browning

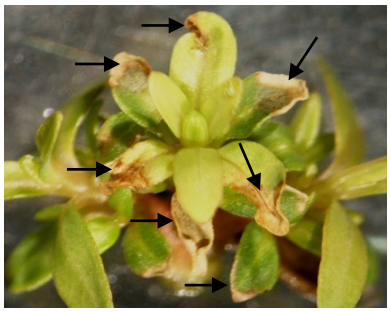
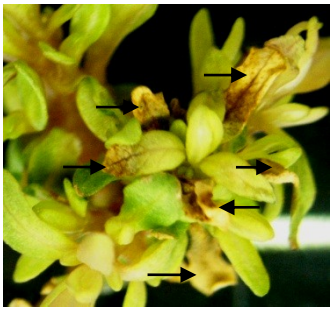
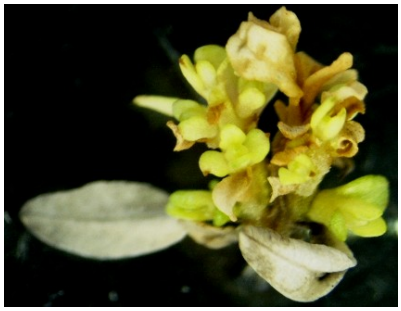
Score	Criteria for score	Visual example of score criteria
0	No visible signs of tissue browning on leaves, stems and axillary buds	
1	Isolated signs of tissue browning on leaves, stem or axillary buds (indicated by black arrows)	
2	Visible signs of tissue browning on leaves and/or stem and/or axillary buds (indicated by black arrows)	
3	Increased signs of tissue browning spread throughout leaves and/or stem and/or axillary buds (indicated by black arrows)	

Table 3. Continued

Severity Score	Criteria for score	Visual example of score criteria
4	Whole shoot cluster displayed widespread browning of tissues but there were still regions of pale and light green living tissues as well	
5	Whole shoot cluster showed tissue browning in its entirety which implied death of shoot	

2.5 Biochemical measurements

Ground shoots (in a pestle and mortar with liquid nitrogen) were used in the assays for phenolics, proline, starch and TSS while whole shoots were directly utilized in the superoxide assay. Preliminary work established that the wet mass of approximately 12-14 shoots was equivalent to 0.1 g. However, when 12-14 hydrated shoots were exposed to cryopreparative drying (Section 2.3.5) the mass after drying was reduced. Therefore, these assays measured immediately (0 h) after drying was based on the mass of the original 0.1 g (fresh wet mass). Furthermore, biochemical assays measured at 12 and 24 h were based on the mass of the rehydrated shoots after being returned onto acclimation medium conditions. Thus, the mass of shoots assayed at 0, 12 and 24 h after drying referred to the original 0.1 g (fresh wet mass) used in the measurements.

2.5.1 Phenolic acid

The procedure of Tabart *et al.* (2007) was used for the extraction of the phenolics from the *E. grandis* material. Phenolics were extracted by the addition of 0.1 g of the ground shoots into 25 ml of 95% ethanol. This was then placed on a shaker (Labcon, South Africa) at 300 revolutions per minute (rpm) in a cold room (8°C) for 3 hours. The concentration of phenolic acid is uniform within the 25 ml of extract, and thus regardless of the volume used for colorimetric quantification the concentration remains the same as within the 25 ml. Post-extraction, 5ml of the extract was centrifuged (Hettich Universal 320, United Kingdom) at 4000 rpm for 15 min. After centrifugation, the supernatant was filtered using Whatman No. 1 Filter Paper to remove remaining residual suspensions. The filtrate was then used in the colorimetric quantification of phenolic acids.

The procedure for the colorimetric determination of phenolics was a combined adaptation from Tabart *et al.* (2007), Torti *et al.* (1995), and Waterman and Mole (1994) and was based on the Folin-Ciocalteau method. The total phenolic acid concentration was represented as a gallic acid (Sigma®) equivalent (GAE) and expressed in the units of mg GAE/g of fresh weight of shoots (FWS). A GAE standard series was established (10-50 $mg.l^{-1}$ at 10 $mg.l^{-1}$ increments) to calculate the content, and at each standard concentration triplicates were measured. From each of the three replicates, 1 mL of the sample filtrate was used in the assay. To the 1 ml filtrate, 1 ml 95% ethanol, 5 ml of water and 0.5 ml of 50% Folin-Ciocalteau (FC) Reagent (Sigma Chemicals) were added. These were thoroughly mixed and allowed to stand for 5 min. This was followed by the addition of 1 ml 5% sodium carbonate and mixing with a

vortex mixer (MRC Model VM-1000, Israel). This sample was then placed in the dark and allowed to stand for 1 h at room temperature. The resultant solution absorbance was measured at 760 nm (Helio Thermo Scientific Spectrophotometer, USA).

2.5.2 Proline

The procedure for the extraction and colorimetric determination of proline was adopted from Gzik (1996), Rohde *et al.* (2004) and Sun *et al.* (2006), which were based on the method described by Bates *et al.* (1973). Proline was extracted by the addition of 0.1 g of ground shoots into 10 ml 3% (w/v) sulfosalicylic acid; the solution was mixed immediately (by a rolling motion between the palms) and centrifuged (Hettich Universal 320, United Kingdom) at 3000 rpm for 5 min. After centrifugation, the supernatant was filtered using Whatman No. 1 Filter Paper. The filtrate was then used in the colorimetric quantification of proline.

Proline concentration was represented as L-proline (Sigma®) and expressed as mg proline.g^{-1} FWS. A proline standard series was established (0.5-8.5 mg.l^{-1} at 1 mg.l^{-1} increments) to calculate the content, and at each standard concentration triplicates were measured. To 2 ml of the sample filtrate from each of the three replicates, 2 ml of freshly-prepared Ninhydrin Reagent and 2 ml of glacial acetic acid was added. The Ninhydrin reagent consisted of 1.25 g ninhydrin (Sigma®) in 30 ml glacial acetic acid. The solution was mixed and incubated at 80°C in the dark for 1 h. After the incubation, the reaction was stopped by dipping the test tubes in cold water. When the contents had cooled, 4 ml toluene was added and this was mixed for 30 seconds on the vortex mixer. The samples were then allowed to stand for 30 min in the dark. The toluene phase was aspirated, and its absorbance measured at 520 nm.

2.5.3 Starch

The colorimetric determination of starch was evaluated by the Megazyme Total Starch Assay Kit (amyloglucosidase/α-amylase). However, the method of *D*-glucose and/or maltodextrins extraction was adapted from Chow and Landhausser (2004) which was based on the technique outlined by Dubois *et al.* (1956) instead of the protocol provided with the kit. Ground shoots (0.1 g) were boiled with 5 ml 80% ethanol at 95°C (Dry Block Heaters, Gemmyco Model DB-006E, China) for 10 min. Before boiling, the suspension was gently mixed using a rolling motion between the palms. After boiling, it was centrifuged (Hettich Universal K2S, United Kingdom) for 10 min at 3000 rpm. The supernatant was decanted into a clean test tube, and the same 0.1 g powder was re-suspended in 80% ethanol and boiled

again for extraction. The supernatants were then collected and combined, and was utilized in the colorimetric determination of total soluble sugars (TSS), while the pellet was used for starch determination.

To the pellet, thermostable α-amylase was immediately added. This was then heated at 100°C (dry block heaters) for 12 min, and gently mixed on a vortex mixer at 4 min intervals. After heating, the sample was transferred to a 50°C water bath (SHZ-82 Water Bath Shaker, China), and then amyloglucosidase was added followed by gentle mixing on a vortex mixer. This was then returned to the water bath for 30 min with gentle shaking. After the 30 min, the sample mixture was centrifuged for 10 min at 3000 rpm and 0.1 ml of the sample was used in the colorimetric test. The extract containing (0.1 ml) was mixed with GOPOD (Glucose oxidase plus peroxidase and 4-amnioantipyrine) reagent, and then incubated for 20 min at 50°C. The absorbance was then measured at 510 nm. Starch content was calculated according to the calculations described in the Megazyme assay procedure of this kit. A more detailed account of the starch assay procedure may be found in the Megazyme Total Starch Assay Procedure (amyloglucosidase/α-amylase).

2.5.4 Total soluble sugars (TSS)

The colorimetric method for TSS determination was adopted from Chow and Landhausser (2004) which was based on the technique outlined by Dubois *et al.* (1956). This method has been specifically adapted for the routine measurement of TSS, namely the hexoses, in woody-plant tissues (Chow & Landhausser, 2004). Extraction for TSS has already been described in Section 2.5.3.

The TSS was represented as a combination of glucose, fructose and galactose (GFG) since these three carbohydrate monomers were determined by Chow & Landhausser (2004) as a suitable reference standard for the approximation of glucose equivalents. Thus, TSS was expressed as mg GFG.g^{-1} FWS. A TSS standard series was established (25-200 mg.l^{-1} at 25 mg.l^{-1} increments) to calculate the content, and at each standard concentration triplicates were measured.

To 1 ml of extract from each of the three replicates, 1 ml 2% (w/v) phenol was added followed by 2.5 ml 95.5% sulfuric acid. In parallel, another set was prepared with water substituting phenol. These were incubated in the dark for 10 min at room temperature, and thereafter cooled in water for 30 min at 22°C to cool down the lukewarm test tubes because

of the exothermic reaction of sulfuric acid with the phenol and sample solutions. The absorbance was measured at 490 nm. The corrected TSS concentration was then determined by the following equation [1]:

$$[sugar]_{corrected} = \frac{A - A'}{a_s - a'_s} \quad [1]$$

A : absorbance with phenol

A' : absorbance without phenol

a_s : absorption coefficient of standards/TSS with phenol

a'_s : absorption coefficient of standards/TSS without phenol

2.5.5 Superoxide

The superoxide production assay was based on the principle of NADH-mediated oxidation of (-)-Epinephrine (Sigma®) to adrenochrome (Beckett *et al.*, 2003; Misra & Fridovich, 1972). 5ml of 1mM epinephrine (in 50 mM HEPES, adjusted to pH 7 with 1 M sodium hydroxide) was added carefully to whole shoots that equalled to the amount of 0.1 g. This was then incubated at 25°C on a shaker (Gallenkamp Orbital Incubator, United Kingdom) at 120 rpm for 15 min in the dark (Beckett *et al.*, 2003; Misra & Fridovich, 1972). Post-incubation, the absorbance of the adrenochrome representative of the superoxide was determined at 480 nm (Beckett *et al.*, 2003; Misra & Fridovich, 1972). The level of the superoxide was calculated by using the adrenochrome molar extinction coefficient of 4020 $M^{-1}cm^{-1}$ (Misra & Fridovich, 1972). It is important to note that the superoxide levels were determined by quantifying the extracellular superoxide produced and released into the external environment of the sampled shoots.

2.6 Light photography

All images presented were viewed with a NIKON stereoscopic zoom microscope (SMZ800) at 10× magnification with a separate light source (Fiberoptic-Heim LQ1100). The images were captured with the NIKON Digital Sight (DS-L1).

2.7 Statistics

Biochemical and physiological results were statistically analysed using the General Linear Model (GLM) that employed univariate tests of significance (factorial-ANOVA), which included subsequent multiple comparisons of results by post-hoc Tukey honest significant

difference (HSD) comparisons at a 0.05 level of significance. It is important to note that when factorial-ANOVA resulted in no significant differences, the subsequent post-hoc Tukey HSD comparisons can indicate significant differences, which was then considered in conjunction with the results of the factorial-ANOVA. When the statistical analysis showed significant differences, the statistical significance was expressed as symbols or letters in figures and tables. The results from *in vitro E. grandis* shoots cultured for 3 days on acclimation or multiplication medium were analysed using the Two-sample Student *t*-Test. The analyses were processed using the programme Statistica 8.0 (StatSoft Inc.), and all analyses were analysed with α at 0.05.

CHAPTER 3.

Understanding some of the fundamental physiological and biochemical responses of *Eucalyptus grandis* cultures to *in vitro* conditions

3.1 INTRODUCTION AND LITERATURE REVIEW

In South Africa the forestry industry relies on clonal plantations, the establishment of which are dependent on both vegetative and *in vitro* methods (see Chapter 1). As the industry wishes to reduce the amount of cultivatable land used for breeding programmes alternative *ex situ* conservation approaches are being considered (see Chapter 1). In this regard cryopreservation of axillary buds obtained from the highly selected and superior clones has been recommended (see Chapter 1). The axillary buds used for cryopreservation could be derived directly from plant material or generated *in vitro*, but in either case the material would have to be regenerated *in vitro* post-cryostorage (Engelmann, 1991 and 2004; Gonzalez-Arnao *et al.*, 2008). Thus, *in vitro* culture is of utmost importance in facilitating the process of plant cryopreservation (Engelmann *et al.*, 2004 and 1991; Padayachee *et al.*, 2009; Watt *et al.*, 2000ab). In brief, *in vitro* culture involves the propagation of selected explants under aseptic conditions, a process also referred to as micropropagation (George, 2008; George & Debergh, 2008). The explants are supplemented with the necessary nutrients (i.e. macro- and micro-nutrients) and a carbon source (e.g. sucrose) in specialised growth media (George & Debergh, 2008; George & de Klerk, 2008; Thorpe *et al.*, 2008), complemented by artificially simulated physical environments (e.g. temperature, photoperiod etc.) that promote growth and development (George & Davies, 2008). As a result, *in vitro* culture provides important advantages (George & Debergh, 2008; Watt *et al.*, 2000ab). One being, the fact that *in vitro* materials are relatively free of diseases and pathogens as they are established from explants that undergo intense decontamination regimes prior to culturing (George & Debergh, 2008; and see Section 2.2.1). Another advantage is the clonal propagation of vegetative materials that can be artificially induced to grow specific plant organ and/or tissue types (e.g. axillary buds and somatic embryos) for cryopreservation (George & Debergh, 2008; and see Section 1.3.2.1). However, there are also some limitations associated with *in vitro* materials, two of which are reduced photosynthetic capacities and stomatal conductance and functioning (Carvalho *et al.*, 2001; George & Debergh, 2008; Saez *et al.*, 2012a,b; Ziv & Chen, 2008).

In order to develop/generate materials (propagules) suitable for cryopreservation, the explants are stimulated by synthetic plant growth regulators (PGRs) that are introduced into the growth medium (Engelmann *et al.*, 2004 and 1991; George & Debergh, 2008; Padayachee *et al.*, 2009). In this study, for example, a ratio of high synthetic cytokinins and low auxin concentrations promoted the multiplication and proliferation of adventitious shoots and buds from *in vitro E. grandis* culture (van Staden *et al.*, 2008; and see Section 2.2.2). Cytokinins are also believed to be involved with the regulation of source/sink relations, especially that of starch and carbohydrates (Gibson, 2004; Roitsch & Ehneβ, 2000; van Staden *et al.*, 2008). Another example also showed that minimal growth storage of *in vitro E. grandis* shoots could be induced by the addition of ABA to growth medium, which undoubtedly slowed growth and allowed the medium-term storage of materials for up to 10 months (Watt *et al.*, 2000b). A further example with *E. grandis* also demonstrated that the concentration, persistence and stability of specific exogenous auxins used to induce *in vitro* root formation subsequently impacted *ex vitro* root development and graviperception (Nakhooda *et al.*, 2011). Thus, specific exogenous PGRs are inducers that subsequently resulted in specific physiological responses.

The uptake of exogenous PGRs by *in vitro* materials from the growth medium therefore induces metabolic changes that influence downstream growth and development (Machakova *et al.*, 2008; Moshkov *et al.*, 2008; van Staden *et al.*, 2008). In the context of this study, metabolic specificity induced by the multiplication-promoting synthetic PGRs for *in vitro E. grandis* shoots could influence the subsequent tolerance responses to low temperature and dehydration stress. Thus, it was necessary to achieve a metabolic state in shoots that is devoid of the influences of synthetic PGRs. This was presently achieved through the culturing of shoots on acclimation medium devoid of PGRs, and allowing the time necessary for metabolic processes of *in vitro E. grandis* shoots to metabolize the residual synthetic cytokinins and auxin (Machakova *et al.*, 2008; van Staden *et al.*, 2008). It was also therefore of particular importance, in this study, to establish the metabolic changes induced by the supplied synthetic PGRs through measuring the biochemical and physiological responses of *in vitro E. grandis* shoots. Similarly, the impact of removal of the PGRs from the medium had to be determined.

3.2 Some physiological and biochemical responses of *in vitro E. grandis* shoots cultured for 3 days on acclimation or multiplication medium

Based on preliminary work in our laboratory, 3 days was selected as the necessary acclimation period for *in vitro E. grandis* shoots.

3.2.1 RESULTS

3.2.1.1 Physiological responses

Water content (WC) of *in vitro E. grandis* shoots cultured on either acclimation or multiplication medium for 3 days were not significantly different (Fig 10). Viability remained 100% over the 2 weeks of evaluation for the shoots cultured on both media, and vigour rates for both the proliferation of axillary buds (emergence of buds) and shoots (growth of shoots) were the same (Figs 11 and 12). There were also no significant differences between the shoots cultured on the two media, in terms of callus formation and tissue browning scores (Table 4).

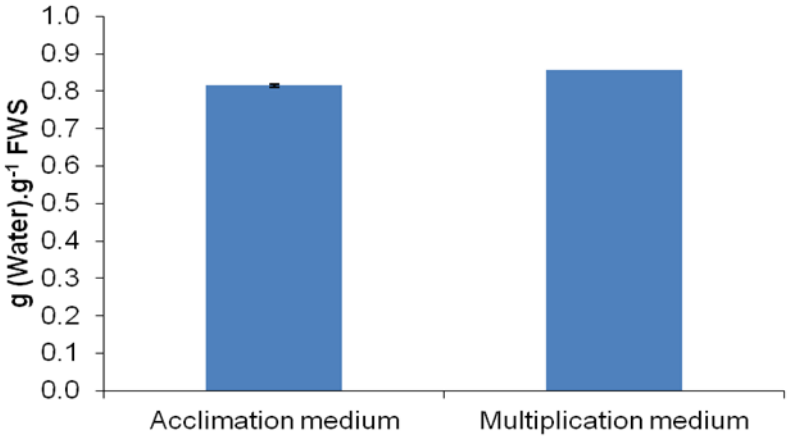


Fig 10. Water content (WC) of *in vitro E. grandis* shoots cultured for 3 days on either acclimation or multiplication medium. Vertical bars represent mean ± SE. There was no significant difference in WC of shoots grown in the two media (two-sample $t(4)= -2.61, p=0.06$).

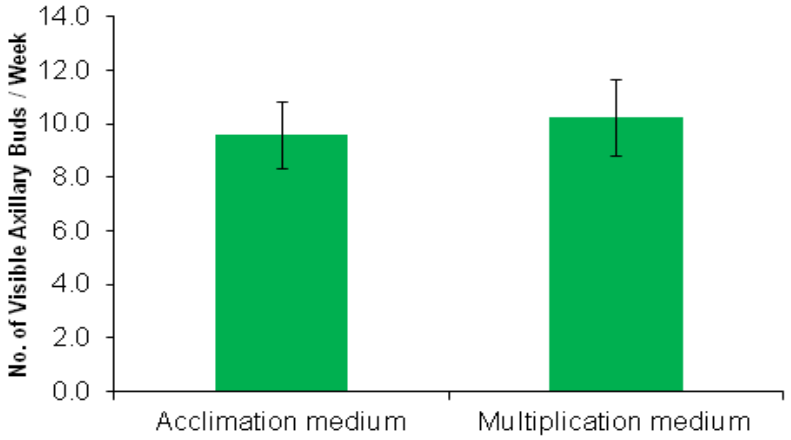


Fig 11. Vigour, in terms of the emergence of axillary buds, of *in vitro E. grandis* shoots cultured for 3 days on either acclimation or multiplication medium. Vertical bars represent mean ± SE. There was no significant difference in vigour of shoots grown in the two media (two-sample $t(4)= -0.35, p=0.74$).

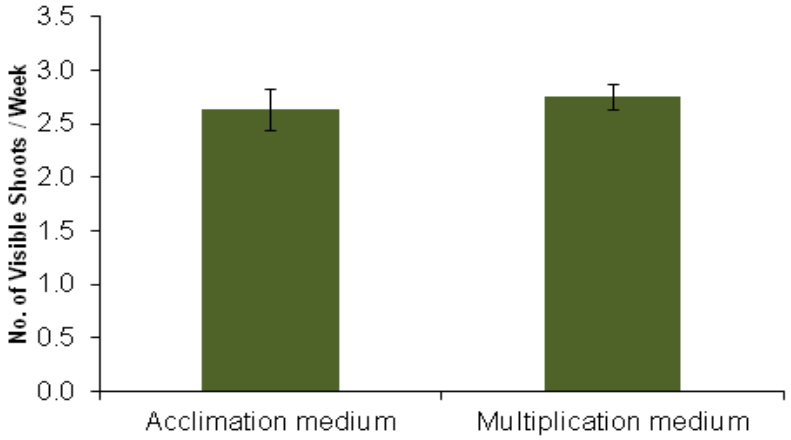


Fig 12. Vigour, in terms of the growth of shoots, of *in vitro E. grandis* shoots cultured for 3 days on either acclimation or multiplication medium. Vertical bars represent mean ± SE. There was no significant difference in WC of shoots grown in the two media (two-sample $t(4)= -0.54, p=0.62$).

Table 4. Callus formation and tissue browning scores evaluated over 2 weeks for *in vitro E. grandis* shoots cultured for 3 days on either acclimation or multiplication medium (with SE). There was no significant difference between the scores for callus formation ($F(1,15)=0.93, p=0.35$) and tissue browning ($F(1,15) = 0.001, p=0.97$).

	Week	Acclimation Medium	Multiplication Medium
Callus Score (0-5)	0	0.0	0.3
	1	0.2 ± 0.2	0.2 ± 0.1
	2	1.0 ± 0.1	1.0 ± 0.1
Tissue Browning Score (0-5)	0	0.4 ± 0.3	0.3
	1	0.4 ± 0.2	0.6 ± 0.2
	2	1.0 ± 0.1	0.9 ± 0.1

3.2.1.2 Biochemical responses

There were no significant differences in the starch and total soluble sugars (TSS), as well as proline levels in *E. grandis in vitro* shoots cultured on either acclimation or multiplication media (Figs 13, 14 and 17). However, phenolic acid and superoxide levels were found to be significantly higher in shoots cultured on multiplication medium (3.11 ± 0.03 mg GAE.g^{-1} FWS and 0.039 ± 0.001 mg adrenochrome.g^{-1} FWS respectively) than shoots cultured on acclimation medium (2.55 ± 0.06 mg GAE.g^{-1} FWS and 0.023 ± 0.001 mg adrenochrome.g^{-1} FWS respectively) (Figs 15 and 16).

Comparison of the proportions of the various biomolecules tested (starch, TSS, phenolic acid, superoxide, and proline) showed that TSS was the highest in shoots irrespective of the medium (Table 5). This was followed by starch and phenolic acid, while on both media proline (higher than superoxide) and superoxide represented less than 1% of the measured biomolecules (Table 5). However, the greatest proportion comprised that of the unknown components, which represented 79.81±1.26% (acclimation medium) and 74.40±3.15% (multiplication medium) (Table 5). The proportion of each biomolecule from shoots cultured on multiplication medium appeared to be higher than that of shoots cultured on acclimation medium (Table 5).

Table 5. Biomolecule proportion within the dry mass of *in vitro E. grandis* shoots cultured for 3 days on either acclimation or multiplication medium (with SE). The table presents the proportion (%) of each class of biomolecules (mg biomolecules.g^{-1} FWS) relative to the mean dry mass of shoots (mg dry mass of shoots.g^{-1} FWS) for each growth medium treatment.

Proportion (%)	Acclimation Medium	Multiplication Medium
TSS	15.38 ± 1.05	19.36 ± 2.70
Starch	3.27 ± 0.41	3.89 ± 0.49
Phenolic Acid	1.46 ± 0.04	2.25 ± 0.02
Superoxide	0.013 ± 0.001	0.028 ± 0.001
Proline	0.059 ± 0.005	0.075 ± 0.009
Unknown	79.81 ± 1.26	74.40 ± 3.15

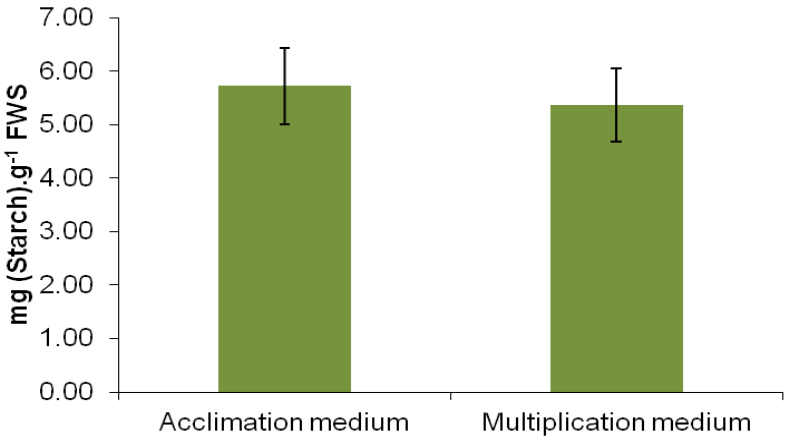


Fig 13. Starch levels of *in vitro E. grandis* shoots cultured for 3 days on either acclimation or multiplication medium. Vertical bars represent mean ± SE. There was no significant difference in the starch levels (two-sample *t(4)=0.35, p=0.74*).

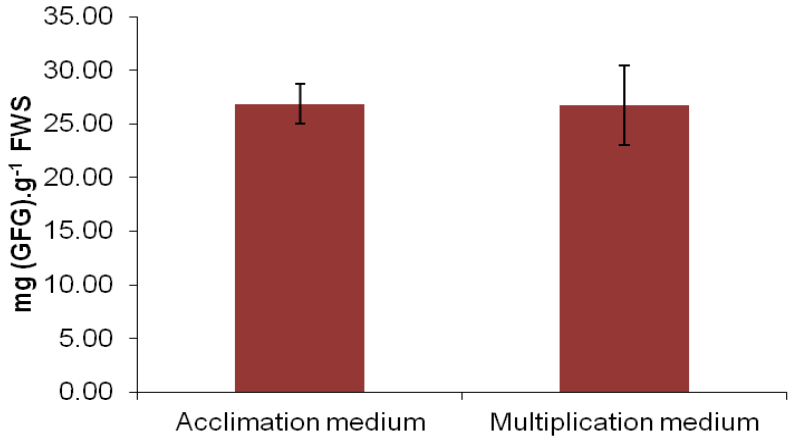


Fig 14. Total soluble sugar, TSS (GFG), levels of *in vitro E. grandis* shoots cultured for 3 days on either acclimation or multiplication medium. Vertical bars represent mean ± SE. There was no significant difference in the TSS levels (two-sample *t(4)=-0.04, p=0.97*).

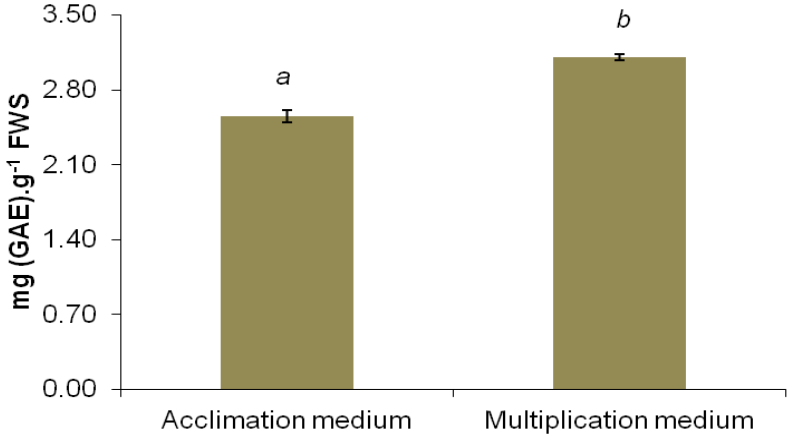


Fig 15. Phenolic acid (GAE) levels of *in vitro E. grandis* shoots cultured for 3 days on either acclimation or multiplication medium. Vertical bars represent mean ± SE. The letters indicate a significant difference in phenolic acid levels (two-sample *t(4)=-8.26, p<0.05*).

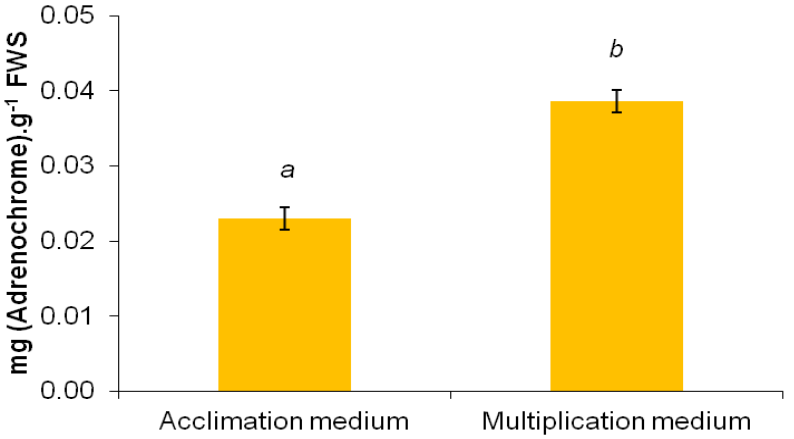


Fig 16. Superoxide levels of *in vitro E. grandis* shoots cultured for 3 days on either acclimation or multiplication medium. Vertical bars represent mean ± SE. The letters indicate a significant difference in superoxide levels (two-sample *t(4)=-7.42, p<0.05*).

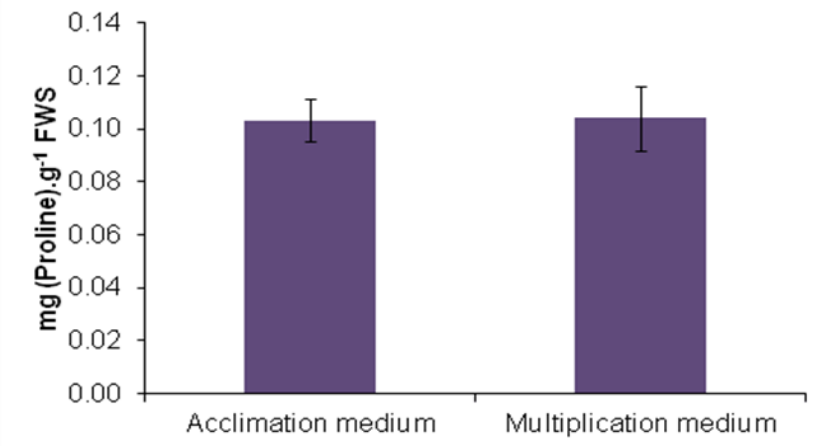


Fig 17. Proline levels of *in vitro E. grandis* shoots cultured for 3 days on either acclimation or multiplication medium. Vertical bars represent mean ± SE. There was no significant difference in proline levels (two-sample $t(4)=-0.05, p=0.96$).

3.2.2 DISCUSSION

The culture of *in vitro E. grandis* shoots on the two media for 3 days did not influence the physiological responses, and the biochemical responses of starch, carbohydrate, and proline (Figs 10-14 and 17, and Table 4). This suggested that the culture period of 3 days in the presence of synthetic PGRs within the multiplication medium may not have been long enough for changes to occur. However, the material cultured for 3 days on multiplication medium had higher phenolic acid and superoxide levels (Figs 15 and 16). It is possible that these higher levels could be attributed to the metabolic induction of increased growth by multiplication-promoting PGRs (see Section 3.1). Increased growth and development would increase the production of the cellular oxidant, superoxide, which would promote the synthesis of antioxidants, phenolic acid (Jones & Hartley, 1999; and see Section 1.5.2.2).

The development stage of the material may have also rendered it more sensitive to light because materials cultured under *in vitro* conditions have reduced photosynthetic capacities (see Section 3.1). This could have resulted in excessive absorbance of light energy, triggering accumulation of phenolic acid for photoprotection (Solecka, 1997; Steyn *et al.*, 2002). In addition, phenolic acid could have contributed as precursors for the cell wall development and promote cell maturation (Solecka, 1997). This phenolic acid accumulation was further promoted in shoots cultured on multiplication medium containing PGRs that promoted growth (Fig 15). Phenolic acid could have also contributed as components of a cytokinin-dependent signalling cascade in cell division and expansion leading to growth as would be expected on a medium designed to stimulate growth and development (Abdulrazzak *et al.*,

2006). In contrast, shoots cultured on acclimation medium for 3 days, devoid of synthetic PGRs, may have depleted the remaining synthetic PGRs that remained in shoots after excision from stock cultures (maintained on multiplication medium). As the PGRs levels decreased in these shoots, metabolism might have become less active, resulting in lower phenolic acid and superoxide levels. However, this would need to be experimentally verified.

From the perspective of the fresh weight of the shoots (FWS), the proportions of starch, soluble carbohydrates and phenolic acid were the prominent biomolecules assayed for *in vitro* shoots, but there were still other unknown biomolecules involved regardless of the type of media (Table 5). However, the detection of these unknown biomolecules was outside the scope of this study. Furthermore, the proportions of each of the tested biomolecules in shoots cultured on multiplication medium were higher (Table 5), further substantiating that the supplied synthetic PGRs promoted metabolism. The dominance of soluble carbohydrates and phenolic acid in metabolism has also been suggested for other *Eucalyptus* (Shvaleva *et al.*, 2006; Warren *et al.*, 2011ab) and plant species (Marron *et al.*, 2002; Sanchez *et al.*, 1998).

3.3 Some physiological and biochemical responses of *E. grandis* shoots cultured for 3, 4 or 6 days on acclimation medium

It should be noted that the 4 and 6 day culture periods form controls for the non-freezing low temperature and exposure (chilling) experiments (Chapter 4). The controls in Chapter 4 are represented as shoots cultured for 3 days on acclimation medium followed by an additional 1 or 3 days on the medium, corresponding with different low temperature exposure periods of 1 or 3 days.

3.3.1 RESULTS

3.3.1.1 Physiological responses

The water contents (WC) of the *in vitro E. grandis* shoots cultured on acclimation medium for 3, 4 and 6 days were almost similar (Fig 18). Viability remained 100% over the 2 weeks of evaluation.

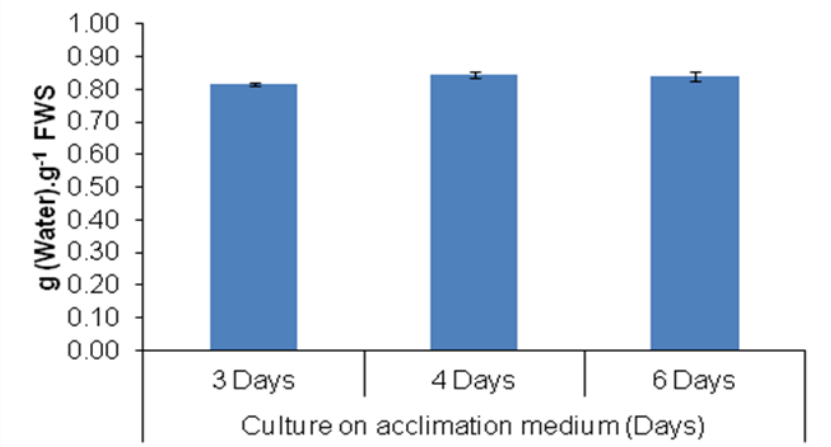


Fig 18. Water content (WC) of *in vitro E. grandis* shoots cultured for 3, 4 or 6 days on acclimation medium. Vertical bars represent mean ± SE. There were no significant differences between the WCs (*F(2,6)=1.58, p=0.28*).

Despite the numeric variation in the measured vigour responses viz. the proliferation of axillary buds (emergence of buds) and shoots (growth of shoots), statistical analyses indicated no significant differences between shoots cultured for 3, 4 or 6 days on acclimation medium (Figs 19 and 20).

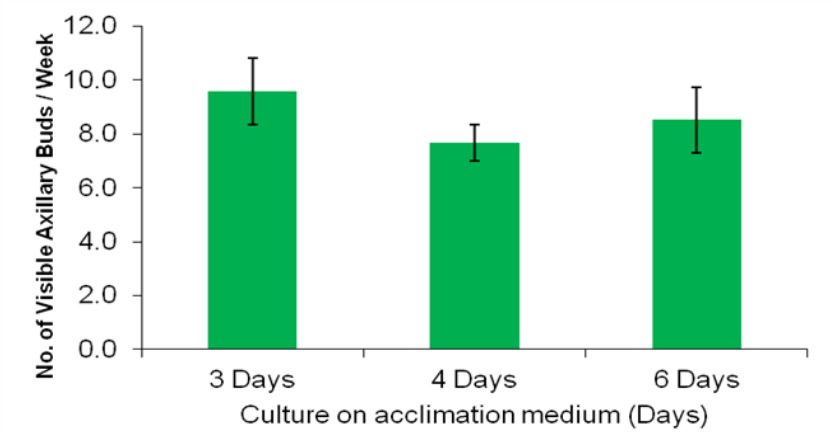


Fig 19. Vigour, in terms of the emergence of axillary buds, of *in vitro E. grandis* shoots cultured for 3, 4 or 6 days on acclimation medium. Vertical bars represent mean ± SE. There were no significant differences in the proliferation rates of axillary buds (*F(2,6)=0.80, p=0.49*).

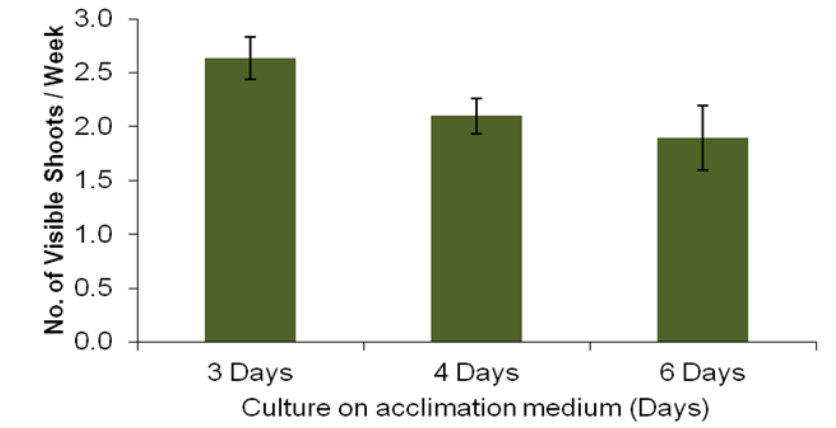


Fig 20. Vigour, in terms of the growth of shoots, of *in vitro E. grandis* shoots cultured for 3, 4 or 6 days on acclimation medium. Vertical bars represent mean ± SE. There were no significant differences in the proliferation rates of shoots (*F(2,6)=2.79, p=0.14*).

Both callus formation and tissue browning were significantly impacted by increasing culture period (Table 6). This was particularly so for materials cultured for 6 days, which was significantly higher than 3 and 4 days on acclimation medium (Table 6).

Table 6. Callus formation and tissue browning scores evaluated over 2 weeks for *in vitro E. grandis* shoots cultured for 3, 4 or 6 days on acclimation medium (with SE). For either callus ($F(2,23)=4.96$, $p<0.05$) scores or tissue browning ($F(2,23)=4.83$, $p<0.05$) severity scores, letters concomitantly indicate significant differences.

	Week	3 Days [a]	4 Days [b]	6 Days [c]
Callus Score (0-5)	0	0.0	0.2 ± 0.1	0.2 ± 0.1
	1	0.2 ± 0.2	0.4 ± 0.2	0.8 ± 0.1
	2	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
Tissue Browning Score (0-5)	0	0.4 ± 0.3	0.5 ± 0.2	0.8 ± 0.1
	1	0.4 ± 0.2	0.9 ± 0.1	1.0 ± 0.0
	2	1.0 ± 0.1	0.9 ± 0.0	1.1 ± 0.0

3.3.1.2 Biochemical responses

3.3.1.2.1 Starch and TSS

Starch and TSS levels from the *in vitro E. grandis* shoots cultured on acclimation medium for 3, 4 and 6 days were not significantly different (Figs 21 and 22).

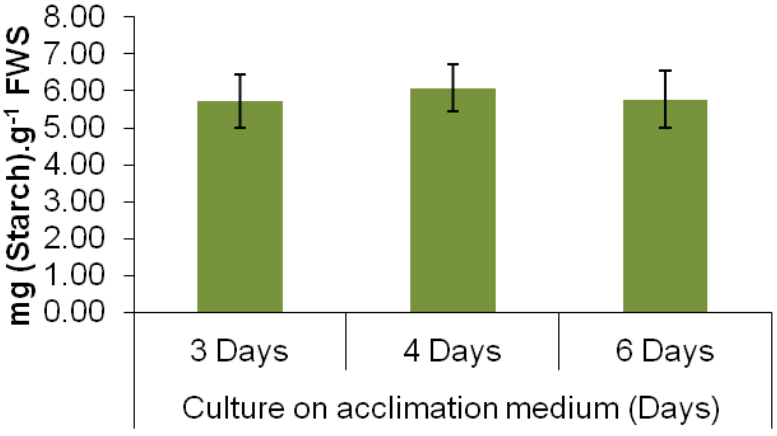


Fig 21. Starch levels of *E. grandis in vitro* shoots cultured for 3, 4 and 6 days on acclimation medium. Vertical bars represent mean ± SE. There were no significant differences in starch levels ($F(2,6)=0.08$, $p=0.92$).

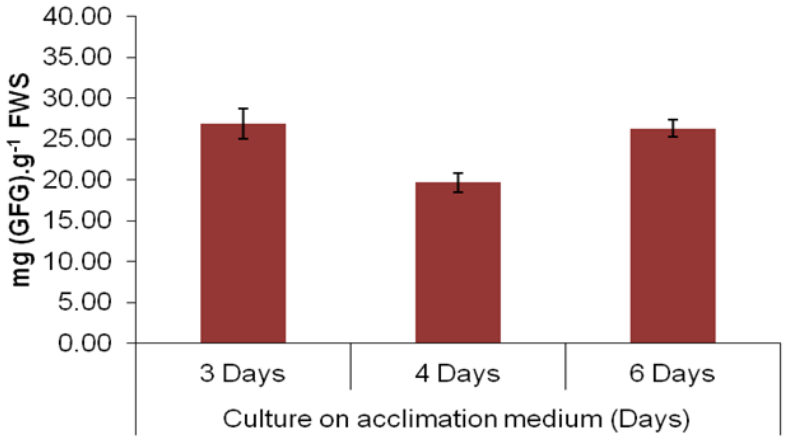


Fig 22. Total soluble sugars, TSS (GFG), levels of *E. grandis in vitro* shoots cultured for 3, 4 and 6 days on acclimation medium. Vertical bars represent mean ± SE. There were no significant differences in TSS levels (F(2,6)=4.65, p=0.06).

3.3.1.2.2 Phenolic acid and superoxide

The phenolic acid levels were significantly different between shoots cultured for 3, 4 and 6 days on acclimation medium (Fig 23). The prominent difference in phenolic acid levels was the decrease after culturing for 4 days. Despite the numeric differences in the mean superoxide levels, there were no significance differences between the levels from the respective culture periods (Figs 23 and 24).

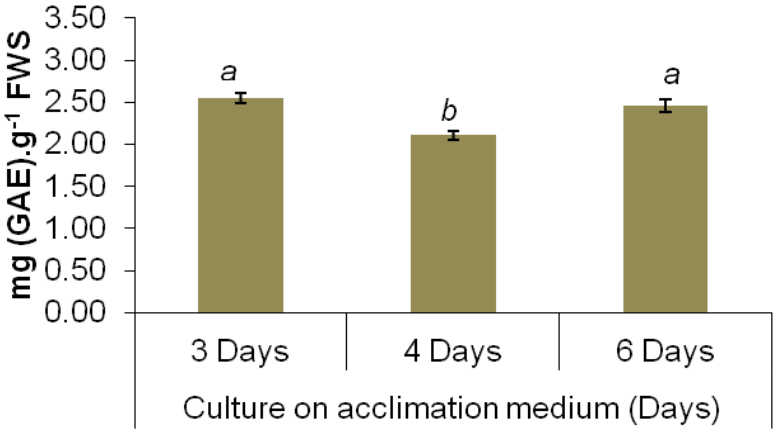


Fig 23. Phenolic acid (GAE) levels of *E. grandis in vitro* shoots cultured for 3, 4 and 6 days on acclimation medium. Vertical bars represent mean ± SE. There letters indicate significance differences (*F(2,6)=12.6, p<0.05*).

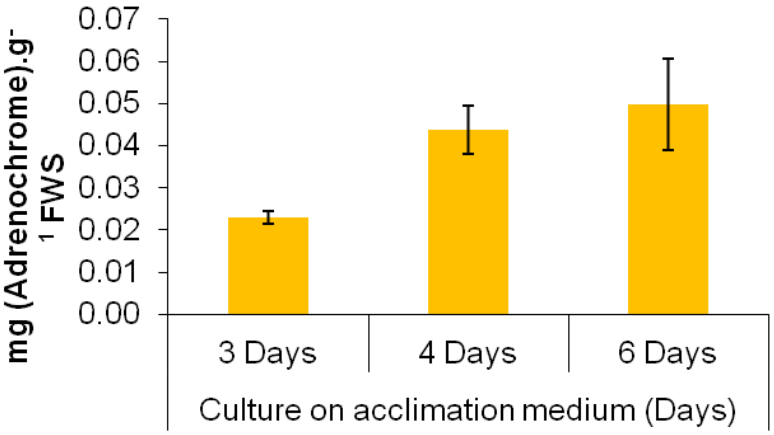


Fig 24. Superoxide levels of *E. grandis in vitro* shoots cultured for 3, 4 and 6 days on acclimation medium. Vertical bars represent mean ± SE. There were no significance differences in superoxide levels (*F(2,6)=3.93, p=0.08*).

3.3.1.2.3 Proline

Proline levels showed a significant increasing trend as culture period increased (Fig 25). Shoots cultured for 6 days contained the highest level (0.179 ± 0.004mg proline.g^{-1} FWS), while no significant difference was noted between shoots cultured for 3 days and 4 days (Fig 25).

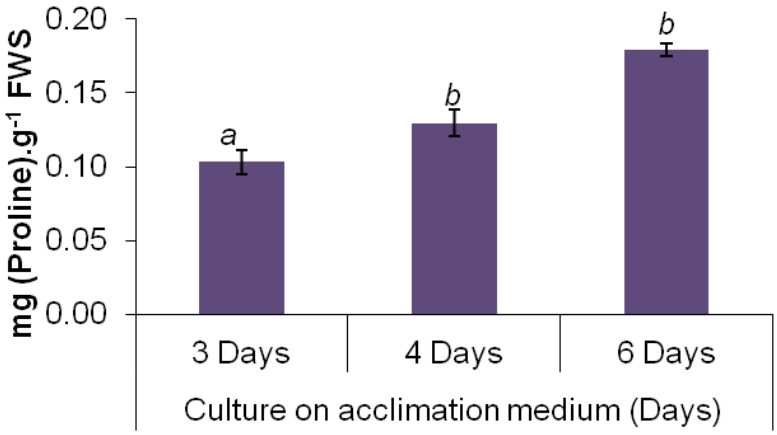


Fig 25. Proline levels of *in vitro E. grandis* shoots cultured for 3, 4 and 6 days on acclimation medium. Vertical bars represent mean ± SE. There letters indicate significant differences (*F(2,6)=26.7, p<0.05*).

3.3.1.2.4 Proportions of biomolecules relative to the fresh weight of shoots (FWS)

A comparison of the proportions of these biomolecules showed that TSS was the highest, followed by starch and phenolic acid, while proline (higher than superoxide) and superoxide represented less than 1% of the dry mass within shoots cultured for 3, 4 and 6 days on acclimation medium (Table 7). Again, the greatest proportion remained that of the unknown components (Table 7).

Table 7. Overall proportion of each class of biomolecule within the dry mass of *in vitro E. grandis* shoots cultured for 3, 4 or 6 days on acclimation medium (with SE). The table represents the proportion (%) of biomolecules (mg biomolecules.g^{-1} fresh weight of shoots, FWS) relative to the mean dry mass of shoots (mg dry mass of shoots.g^{-1} FWS) for each treatment.

Proportion (%)	3 Days	4 Days	6 Days
TSS	15.38 ± 1.05	13.24 ± 0.76	17.59 ± 2.33
Starch	3.27 ± 0.41	4.08 ± 0.42	3.85 ± 0.05
Phenolic Acid	1.46 ± 0.04	1.42 ± 0.04	1.64 ± 0.05
Superoxide	0.013 ± 0.001	0.029 ± 0.003	0.033 ± 0.007
Proline	0.059 ± 0.005	0.087 ± 0.006	0.120 ± 0.003
Unknown	79.81 ± 1.26	81.15 ± 1.14	76.76 ± 2.34

3.3.2 DISCUSSION

The stock cultures, from which the experimental material was obtained, were maintained on multiplication medium, and these would have absorbed the synthetic PGRs from the medium, especially the predominant synthetic cytokinins in the medium. As the experimental culturing period on acclimation medium (without PGRs) increased, the residual synthetic PGRs may have slowly depleted. Metabolism may have slowed as residual synthetic PGRs became depleted after 4 days of culture, hence the statistically lower phenolic acid level (Fig 23). In contrast, the other biomolecules showed no significant changes in levels between shoots cultured for the different periods, despite the numeric differences in levels (Figs 21, 22, and 24). However, these biochemical responses along with the proportions of these biomolecules indicated that the biomolecules phenolic acid, proline and soluble carbohydrates (soluble sugars, TSS) were active throughout the increasing culture periods, but the high unknown components indicated that there were other biomolecules involved as well (Table 7), where

the peak of some biochemical and physiological responses had occurred only after 6 days of culture.

Only after 6 days of culture had growth-related metabolism returned in shoots, which could be attributed to the synthesis of endogenous plant growth regulators. However, this would need to be experimentally verified for *in vitro E. grandis* shoots, since it is believed that many small tissues and organs developed *in vitro* may not synthesise the necessary levels of natural cytokinins for sustained growth (van Staden *et al.*, 2008). However, the significantly higher level of proline and phenolic acid after 6 days culture (see Section 3.3.1; Figs 23 and 25) could substantiate the return of growth-related metabolism not promoted by synthetic PGRs. The stimulation of proline synthesis was presumably necessary for protein synthesis and for growth and development. Under growth conditions, amino acids including proline are synthesised by plant cells for sustained developmental programmes of rapid cell growth such as in cell and stem elongation (Lehmann *et al.*, 2010; Mattioli *et al.*, 2009; Trovato *et al.*, 2008; Verbruggen & Hermans, 2008). In protein synthesis, proline is the building block for the synthesis of hydroxyproline-rich glycoproteins, which are important structural constituents of the plant cell wall that are thought to be key role players in the regulation of cell division and the self-assembly of cell walls (Lehmann *et al.*, 2010; Mattioli *et al.*, 2009; Showalter, 1993; Trovato *et al.*, 2008; Verbruggen & Hermans, 2008). Proline has also been proposed as an alternative energy source for sustaining high energy-requiring processes of rapid plant development and growth, since the oxidation of proline can yield 30-ATP equivalents higher than that of a hexose (Mattioli *et al.*, 2009; Trovato *et al.*, 2008). In addition, proline content is also dependent on plant age, and the age, position and part of the leaf (Hua *et al.*, 1997; Lehmann *et al.*, 2010; Verbruggen & Hermans, 2008). For example, evidence from young *Arabidopsis* plants indicated that there was high accumulation of proline in the apical meristem and young leaf (Hua *et al.*, 1997).

Finally, the increased metabolic activities after 6 days of culture was further supported by the significantly high callus formation and tissue browning (Table 6), which could be regarded as physiological manifestations of fluctuations of metabolism. Water content (WC) and growth (emergence of axillary buds and growth of shoots), on the other hand, were not affected (Figs 18-20). The proportions of each biomolecules further substantiated that metabolic activities were high after 6 days of culture, as there were active re-allocations of proportions from the unknown components and starch (Table 7). However, the differences observed in shoots

cultured on acclimation medium at increasing durations needs to be experimentally verified through PGR and further metabolic and photosynthetic analyses.

3.4 SUMMARY AND CONCLUSION

The culturing of *in vitro E. grandis* shoots for 3 days on multiplication and acclimation media resulted in similar physiological responses and, in most part, biochemical responses (Figs 10-14 and 17, and Tables 4 and 6). The biochemical changes that occurred were increased phenolic acid synthesis and superoxide production in shoots cultured on multiplication medium (Figs 15 and 16) These biochemical changes may be attributed to the multiplication-orientated metabolism induced by high cytokinins and low auxin concentrations, whereas at a physiological perspective, the culturing of 3 days may not have been long enough to induce any developments (see Section 3.2). When shoots were cultured on acclimation medium for 3, 4 and 6 days, changes in biochemical responses only significantly occurred after 6 days, which was characterised by a high proline level and increased phenolic acid content (Figs 23 and 25). These biochemical changes may be attributed to the endogenous synthesis of PGRs inducing metabolic responses, since residual synthetic PGRs may have become slowly metabolised and depleted after 3 days culture on acclimation medium. This would however have to be experimentally verified (see Section 3.3). These data show that exogenous synthetic PGRs can influence the biochemical responses of *in vitro E. grandis* shoots. They also validate the necessity for the use of the acclimation medium, which is devoid of synthetic PGRs, in order to achieve a metabolic state that is not specified through the induction of synthetic cytokinins and auxin. This therefore eliminated the possibility of synthetic PGRs affecting the tolerance responses of shoots to low temperature and dehydration stress that are subsequently investigated.

CHAPTER 4.

Understanding some of the physiological and biochemical responses of *in vitro Eucalyptus grandis* shoots to different non-freezing low temperatures and culture periods

4.1 INTRODUCTION AND LITERATURE REVIEW

The underlying concept of cellular acclimation is supported by various studies (Table 8). This concept involves the prior exposure of plants to an abiotic strain, which may trigger and induce metabolic adjustments that could prepare the plants to tolerate and survive subsequent encounters of abiotic stress. An example of this is that of cold acclimation (or cold hardiness on a whole plant scale), which involves prolonged exposure of vegetative plants to chilling conditions that results in improved tolerance towards frost (Gaspar *et al.*, 2002; Nilsen & Orcutt, 1996). The chilling temperatures suggested for promoting acclimation and hardening have been suggested to range between 5°C and 10°C, and are believed to increase freezing (e.g. frost) tolerance in vegetative plants (Chalker-Scott, 1999). This acclimation and hardening brings about adaptive metabolism that includes the accumulation of protective biomolecules such as soluble carbohydrates, proline, and phenolic acid (see Section 1). The success of cold acclimation is well-documented for a variety of different species including some eucalypts (Table 8).

Table 8. Examples of non-freezing low temperatures and culture periods employed for cold acclimation from different *Eucalyptus*, woody and non-woody species. Arrows (→) indicate changes in non-freezing low temperatures from the initial low temperature and the additional days of culturing at the new temperatures, which are part of the cold acclimation process.

Species	Type of material	Chilling condition		Culture period (Days)	Reference
		Day (°C)	Night (°C)		
Eucalyptus species					
Eucalyptus globulus (clones CN5 and ST51)	Rooted cuttings	24 to 10 (1/day)	16 to 6 (1/night)	14	Costa E Silva *et al.*, 2008
E. gunnii × *E. dalrympleana* (transgenic lines)	*In vitro* plantlets	12→4	8→4	4→6	El Kayal *et al.*, 2006
E. gunnii (941366)	3-year-old plantlets	12→4	8→4	4→12	Keller *et al.*, 2009

Table 8. Continued

E. urophylla × *E. grandis* (transgenic lines)	*In vitro* shoot clumps	12→8→4	8→4→4	4→7→7	Navarro *et al.*, 2011
E. globulus Labill. (CN5 and ST51)	Rooted cuttings	24 to 10 (1.4/day)	16 to 5 (1.4/night)	10	Shvaleva *et al.*, 2008
E. grandis Hill (ex. Maiden)	*In vitro* shoots	12	12	½	Souza *et al.*, 2004
E. nitens and *E. pauciglora*	Seedlings	10-25	4	98	Warren *et al.*, 1998
Woody species					
Pyrus paisha and *P. cordata*	6-week old *in vitro* shoots	4	4	7	Chang & Reed, 2000
Rubua idaeus L.	*In vitro* plantlets	15→2→ -2→2	15→2→ -2→2	14→14 →1→3	Palonen *et al.*, 2000
Pinus sylvestris L.	1-year-old seedlings	15→5	10→5	21→21	Sveshinkov *et al.*, 2006
Non-woody species					
Lolium perenne (cv. Arka)	Seedlings	4	2	21	Bocian *et al.*, 2011
Brassica napus L.	*In vitro* seedlings	4	4	7, 14 or 21	Burbulis *et al.*, 2011
Arabidopsis thaliana (Columbia)	2-week old plants 12/14-day-old seedlings	4	4	7 or 3	Cuevas *et al.*, 2008
T. aestivum (cv. Quantum)	7-day-old seedlings	4	4	1, 3 or 6	Farajalla & Gulick, 2007
A. thaliana (Wassilewskija-2)	Seedlings	4	4	1 or 7	Fowler & Thomashaw, 2002

Table 8. Continued

Trifolium and *Medicago* spp. (legumes)	Seedlings	10	5	24-28	Hekneby *et al.*, 2006
Triticum aestivum L. (cv. Cheyenne and Chinese Spring)	10-day-old hydroponic seedlings	5	5	2	Horvath *et al.*, 2007
Solanum tuberosum, *S. acaule* and *S. demissum*	*In vitro* shoots	22	8	7	Kaczmarczyk *et al.*, 2008
A. thaliana (Columbia)	Seedlings	4	4	1, 2 or 4	Kaplan *et al.*, 2007 and 2004
T. aestivum (cv. Chinese Spring)	Hydroponic whole shoots	2	2	1, 3 or 7	Kovacs *et al.*, 2011
Hordeum vulgare L. (accessions)	Hydroponic seedlings	4	4	2, 7 or 21	Limin *et al.*, 2007
A. thaliana (wild-type and transgenic)	3-week-old plants	4	4	1 or 4	Maruyama *et al.*, 2009
T. aestivum (cultivars)	Seedlings	4	4	1, 3 or 6	Monroy *et al.*, 2007
A. thaliana L. Heyhn (Columbia)	Seedlings	2	2	2 or 7	Nagao *et al.*, 2008
A. thaliana (Columbia)	3-week-old plants	4	4	1, 2 or 4	Oono *et al.*, 2006

Acclimation may not only be limited to low temperature stress, but may also be plausible across different abiotic stresses, as inferred from molecular evidences of cross-talk (see Section 1.5.3, and Table 8), since one of the consequences of freezing, like frost in the natural

environment, can lead to desiccation of plant cells (see Section 1.4.3). This may mean that the same chilling conditions of cold acclimation may influence resistance towards different abiotic stress factors, such as dehydration. In the context of this study, pre-exposure to a specific non-freezing low temperature and culture period (chilling condition) could promote acclimation towards physical drying i.e. cross-acclimation or cross-resistance, as described by Chalker-Scott (1999).

The appropriate chilling condition for acclimation has to be specifically determined for the species and material of choice, especially in this regard is the plant's natural temperature requirements. Cold acclimation conditions are therefore variable across different species and material types (Table 8). Temperate and hardier species and older seedlings appear to require longer culture periods at non-freezing low temperatures, whereas tropical or sub-tropical species such as *E. grandis* require shorter culture periods to promote acclimation (Table 8). In some instances, especially with temperate eucalypts and plant species, acclimation is also achieved through simulating day-night temperature conditions similar to seasonal and/or daily fluctuations (i.e. from autumn to winter), which involve a progressive decrease of temperatures at prolonged or shortened culture periods and decreasing non-freezing low temperatures (Table 8). However, what is equally important for the selection of an appropriate chilling condition is an understanding of the physiological and biochemical responses of the vegetative material to chilling, which then provides an indication as to which of the conditions are most appropriate.

The natural mean annual temperature preference of *E. grandis* is within the range of 14°C and 25°C, where the mean minimum temperature of the coldest month is between 3°C and 16°C (Booth & Pryor, 1991). Thus, based on literature (Table 8) and the natural temperature conditions of *E. grandis*, the non-freezing low temperatures of 5°C, 10°C and 15°C were selected, since these remained within its natural range. The culture period for *in vitro* shoots at these temperatures were either 1 or 3 days, based on inferences from literature (Table 8). These culture periods were also selected based on the reality that *E. grandis* is a sensitive sub-tropical species, and like cold acclimation experiments with the model plant *A. thaliana*, would be more responsive to shorter culture periods, compared with temperate eucalypts and plant species (Table 8). In addition, previous work in our laboratory had indicated that culturing of shoots at 4°C for 5 days had a physically negative impact on its physiology (unpublished). Thus, the aforementioned chilling conditions suggested in this study may strain *in vitro E. grandis* shoots and trigger adaptive metabolism, but importantly not cause

stress. Little is specifically known about the physiological and biochemical responses of *in vitro E. grandis* shoots to these conditions. It was therefore important to understand these responses, in order to assist in the selection of an appropriate chilling condition (specific non-freezing low temperature and culture period).

4.2 RESULTS

The shoots were cultured for 3 days on acclimation medium and then transferred to the selected non-freezing low temperatures of 5, 10 or 15°C for 1 or 3 days.

4.2.1 Physiological responses

The water content (WC) of the *in vitro E. grandis* shoots showed no significant differences between the different chilling treatments and with the controls (Fig 26).

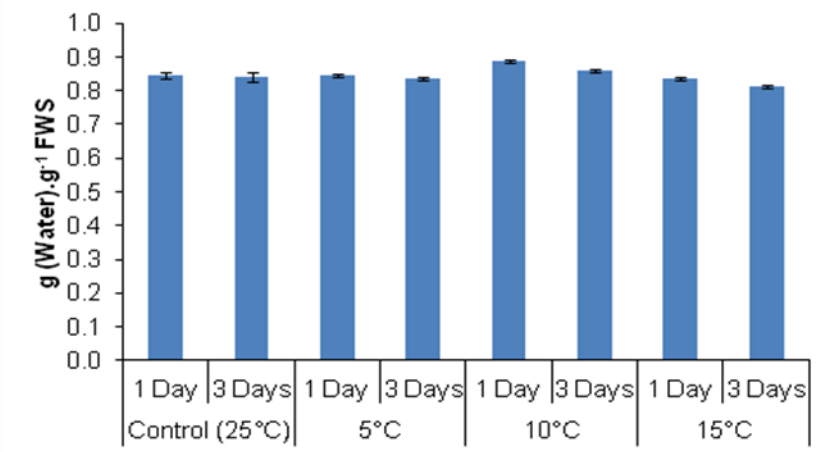


Fig 26. Water content (WC) of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days. Vertical bars represent mean ± SE. There was no significant differences according to post-hoc Tukey HSD (0.05 level of significance, *F(3,16)=1.3, p=0.32*).

The viability of the *in vitro E. grandis* shoots, exposed to the different chilling treatments, all remained 100% after the 2 week evaluation period (Table 9). The 10°C treatment, irrespective of culture period, demonstrated the highest vigour, while the other treatments remained similar to that of the control (25°C) (Figs 27 and 28). However, this was only statistically supported for vigour in terms of growth of shoots, where growth from shoots treated at 10°C for 3 days were significantly higher than the other treatments (Fig 28). Vigour in terms of the emergence of axillary buds, however, showed no significant differences (Fig 27).

Table 9. Viability of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days

		Control (25°C)		5°C		10°C		15°C	
	Week	1 Day	3 Days	1 Day	3 Days	1 Day	3 Days	1 Day	3 Days
Viability (%)	0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

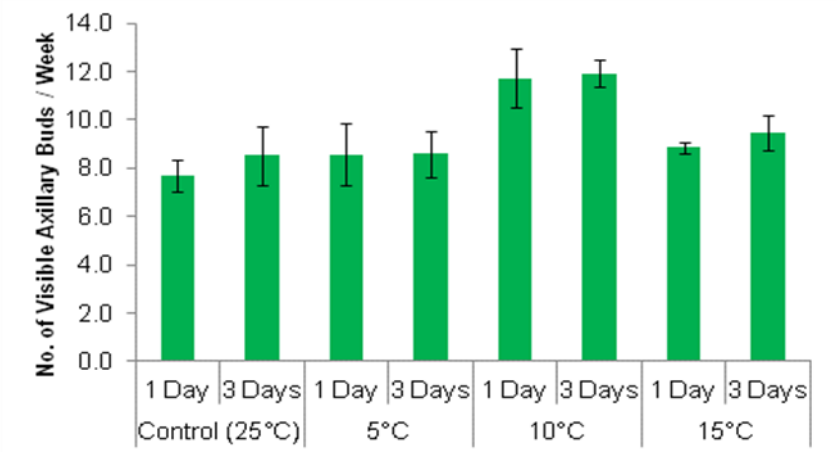


Fig 27. Vigour, in terms of the emergence of axillary buds, of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days. Vertical bars represent mean ± SE. There were no significant differences (*F(3,16)=0.11, p=0.96*).

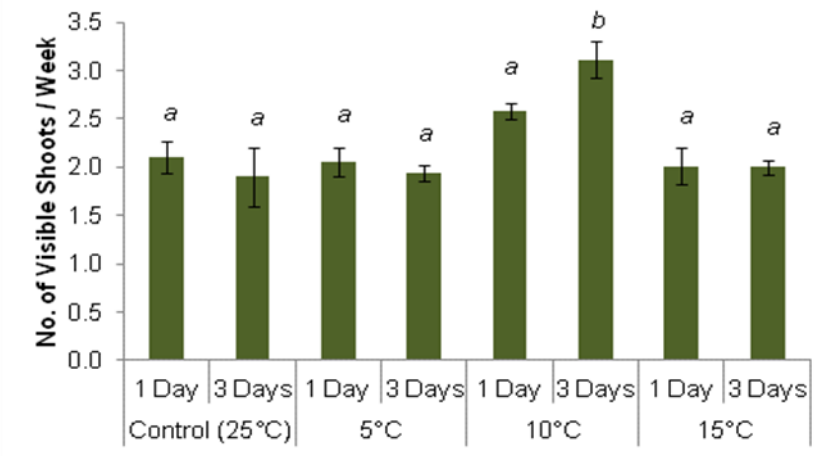


Fig 28. Vigour, in terms of the growth of shoots, of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days. Vertical bars represent mean ± SE. The letters indicate significant differences, according to post-hoc Tukey HSD (0.05 level of significance, *F(3,16)=1.97, p=0.16*).

Callus formation scores were significantly different between the chilling treatments and controls. The callus scores from the 15°C, 3 day treatment (lowest of all treatments) and the 10°C, 1 day treatment were lower than the control (25°C) at 3 days, while the remaining treatments were similar (Table 10). On the other hand, tissue browning scores were not significantly different between the chilling treatments and controls, even though scores from 10°C were the lowest of the chilling treatments and were similar to the 1 day control (Table 10).

The morphological effects of the different chilling treatments on *in vitro E. grandis* shoots are presented in Table 11. At week 0, shoot growth from both controls were more prominent than that of the shoots exposed to the different chilling treatments (Table 11). In addition, the stems of the treated shoots at week 0 showed reddish pigmentation, which was more prominent with shoots that were exposed to the 3 day chilling treatments (Table 11). After the second week, the appearances of the shoots were indistinguishable across the control and chilling treatments (Table 11).

Table 10. Callus formation and tissue browning scores of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days (with SE). The letters indicate significant differences of scores for callus formation between the different chilling treatments and controls, according to post-hoc Tukey HSD (0.05 level of significance, $F(3,63)=4.44,\ p<0.05$). There were no significant differences noted between the scores for tissue browning ($F(3,63)=0.59,\ p=0.62$).

Score		Control (25°C)		5°C		10°C		15°C	
(0-5)	Week	1 Day	3 Days	1 Day	3 Days	1 Day	3 Days	1 Day	3 Days
Callus Formation	0	0.2 ± 0.1^{a}	0.2 ± 0.1^{b}	0.1 ± 0.1^{a}	0.1 ± 0.0^{a}	0.0 ± 0.0^{c}	0.0 ± 0.0^{a}	0.3 ± 0.1^{a}	0.0 ± 0.0^{d}
	1	0.4 ± 0.2^{a}	0.8 ± 0.1^{b}	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	0.4 ± 0.1^{c}	0.4 ± 0.1^{a}	0.6 ± 0.2^{a}	0.1 ± 0.0^{d}
	2	1.0 ± 0.1^{a}	1.2 ± 0.1^{b}	0.9 ± 0.0^{a}	1.0 ± 0.0^{a}	0.8 ± 0.2^{c}	0.9 ± 0.0^{a}	0.9 ± 0.2^{a}	0.8 ± 0.2^{d}
Tissue Browning	0	0.5 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.3 ± 0.3	0.3 ± 0.2	0.8 ± 0.1	0.8 ± 0.0
	1	0.9 ± 0.1	1.0 ± 0.0	0.9 ± 0.1	1.0 ± 0.0	0.6 ± 0.2	0.8 ± 0.1	1.0 ± 0.0	1.0 ± 0.0
	2	0.9 ± 0.0	1.1 ± 0.0	0.9 ± 0.1	1.0 ± 0.1	0.6 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	1.2 ± 0.0

Table 11. Representative examples of *in vitro E. grandis* shoots (10× magnification) 0 and 2 weeks after exposure to 5°C, 10°C or 15°C for 1 or 3 days. The shoots from week 0 were photographed when the shoots were still within culture tubes, and week 2 shoots were photographed after removal from culture tubes. The scale for week 0 is 10mm:0.61mm, and week 2 is 10mm:0.88mm.

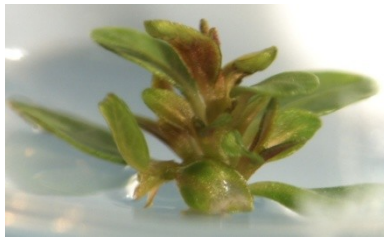
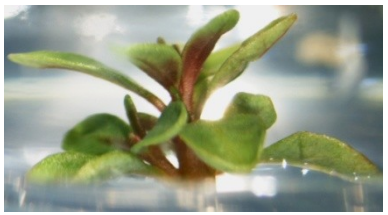
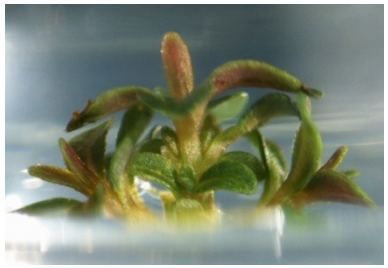
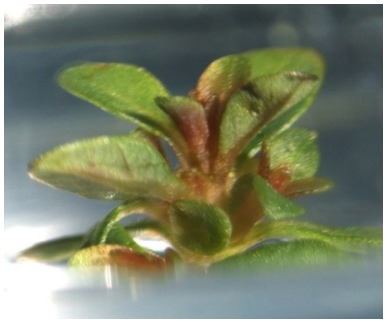
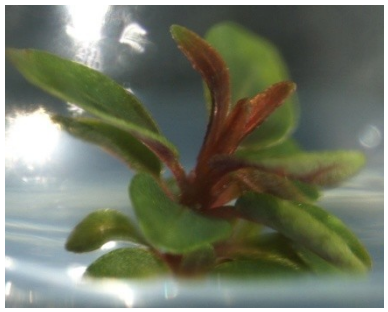
Week	Exposure (Days)	Control (25°C)	5°C	10°C	15°C
0	1				
	3				

Table 11. Continued

Week	Exposure (Days)	Control (25°C)	5°C	10°C	15°C
2	1				
	3				

4.2.2 Biochemical responses

4.2.2.1 Starch and TSS

The starch levels from *in vitro E. grandis* shoots showed no significant differences between the levels extracted from material grown in the different chilling treatments and the controls. However, starch levels after 5°C treatment were numerically lower than the controls, 10°C and 15°C treatments (Fig 30). Similarly, the level after the 3 day treatment at 15°C was the numerically highest (7.69 ± 0.71 mg starch.g^{-1} FWS) followed by the level after 3 day treatment at 10°C (7.11 ± 0.74 mg starch.g^{-1} FWS) (Fig 29).

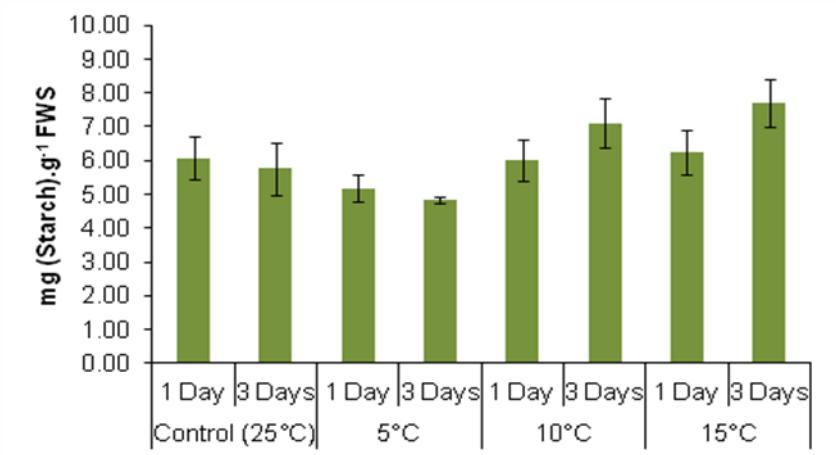


Fig 29. Starch levels of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days. Vertical bars represent mean ± SE. There were no significant differences (*F(3,16)=1.16, p=0.36*).

Total soluble sugar (TSS) in shoots after 3 days exposure was greater than after 1 day exposure throughout all the chilling treatments and controls (Fig 30), but there were no significant differences between the treatments. The levels after 1 day exposure at 5°C, 10°C and 15°C were similar to the 1-day control, while levels after 3 day exposure at 5°C and 10°C were similar to the 3-day control (Fig 30). Numerically, the highest TSS level was only observed after 3 days at 15°C (40.55 ± 4.59 mg GFG.g^{-1} FWS), followed by the levels after the 10°C, 3 day treatment (30.61 ± 1.51 mg GFG.g^{-1} FWS) (Fig 30).

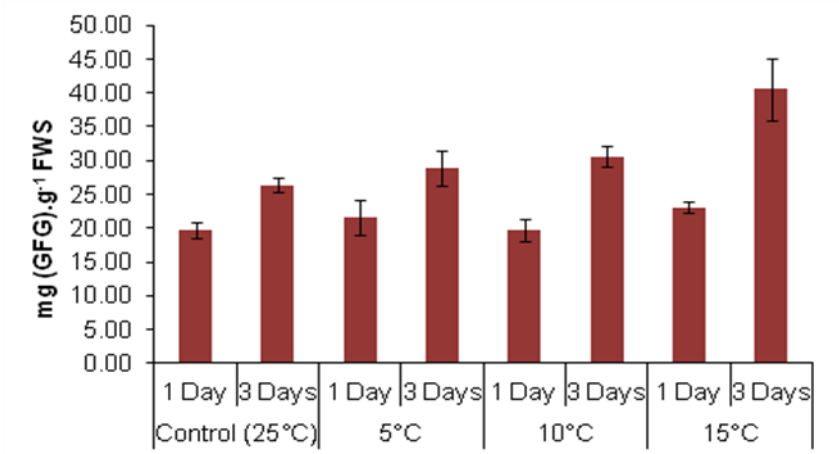


Fig 30. Total soluble sugars (TSS) levels of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days. Vertical bars represent mean ± SE. There were no significance differences (*F(3,16)=1.40, p=0.28*).

4.2.2.2 Proline

Proline accumulation in shoots was numerically highest in the 3-day control (0.179 ± 0.004 mg proline.g^{-1} FWS) and in the material incubated at 5°C for 3 days (0.162 ± 0.009 mg proline.g^{-1} FWS) (Fig 31). All other treatments were the same as the 1-day control (Fig 31).

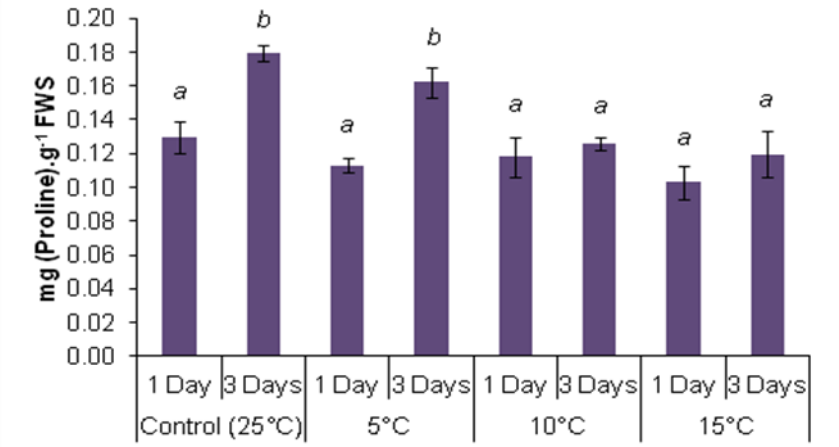


Fig 31. Proline levels of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days. Vertical bars represent mean ± SE. The letters indicate significant difference, according to post-hoc Tukey HSD (0.05 level of significance, *F(3,16)=2.92, p=0.07*).

4.2.2.3 Phenolic acid and superoxide

Phenolic acid levels from *in vitro E. grandis* shoots showed significant differences between the different chilling treatments and controls. The material from the 3-day control and all of the chilling treatments had higher phenolic acid levels than the 1-day control (Fig 32). Phenolic acid accumulation in shoots was significantly the highest after being treated at 10°C for 3 days (3.05 ± 0.09 mg GAE.g^{-1} FWS), while other treatments remained similar to each other and the 3-day control (Fig 32).

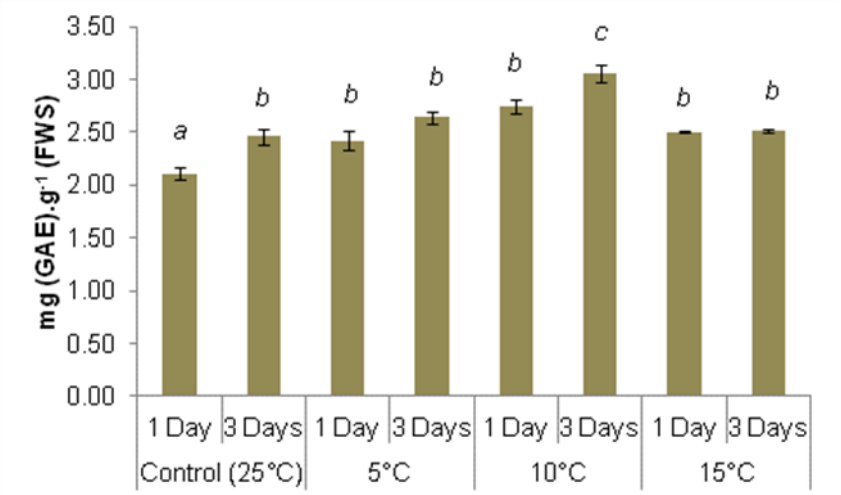


Fig 32. Phenolic acid (GAE) levels of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days. Vertical bars represent mean ± SE. The letters indicate significant differences, according to post-hoc Tukey HSD (0.05 level of significance, $F(3,16)=3.59, p<0.05$).

The highest superoxide level was assayed from shoots exposed to 5°C for 3 days 0.091±0.003mg adrenochrome/g FWS) (Fig 33). The material in the remaining chilling treatments had superoxide levels similar to the respective controls (Fig 33), although there was a numeric decrease in superoxide levels in shoots cultured for 3 days at 15°C (Fig 33).

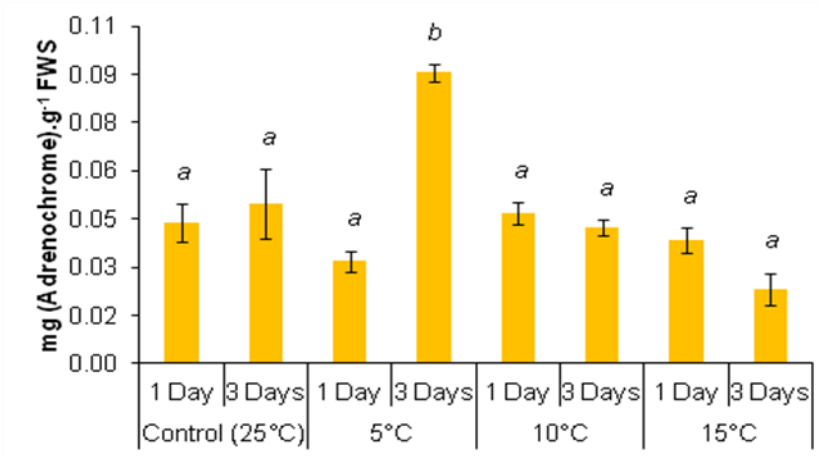


Fig 33. Superoxide (adrenochrome) of levels of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days. Vertical bars represent mean ± SE. The letters indicate significant differences (*F(3,16)=19.36, p<0.05*).

When the phenolic acid and superoxide levels were considered together, it was noted that there were increased levels of phenolic acid in shoots held at 10°C and 15°C whilst superoxide levels remained similar to their controls (Figs 32 and 33). However, this was not equally reflected from shoots that were treated at 5°C for 3 days, where phenolic acid and superoxide levels increased (Figs 32 and 33).

4.2.2.4 Proportions of biomolecules relative to the fresh weight of shoots (FWS)

A comparison of the proportions of the tested biomolecules showed that in shoots exposed to 10°C starch and phenolic acid were the highest (Table 12), and together with TSS the proportions reduced the unknown components to within the 70-75% range (Table 9). After the shoots were cultured for 3 days at 10°C, the proportion of each biomolecule decreased compared with proportions after 1 day, with the exception of TSS (Table 12). Shoots treated for 3 days at 15°C also showed higher TSS and starch proportions (Table 12). The unknown component proportions for the controls and all chilling treatments were lower after 3 days when compared with the proportions after 1 day (Table 12), whereas after the 3 day culture under those conditions, TSS proportions showed the greatest increase (Table 12).

Table 12. Overall proportion of each class of biomolecule within the dry mass of *in vitro E. grandis* shoots exposed to 5°C, 10°C or 15°C for 1 or 3 days (with SE). The table represents the proportion (%) of biomolecules (mg biomolecules.g^{-1}fresh weight of shoots, FWS) relative to the mean dry mass of shoots (mg dry mass of shoots.g^{-1}FWS) for each treatment.

Proportion (%)	Control (25°C)		5°C		10°C		15°C	
	1 Day	3 Days	1 Day	3 Days	1 Day	3 Days	1 Day	3 Days
TSS	13.24 ± 0.76	17.59 ± 2.33	14.32 ± 1.67	18.04 ± 1.61	17.99 ± 1.46	21.87 ± 1.08	14.14 ± 0.47	22.55 ± 2.55
Starch	4.08 ± 0.42	3.85 ± 0.05	3.44 ± 0.27	3.03 ± 0.05	5.46 ± 0.56	5.08 ± 0.53	3.82 ± 0.41	4.28 ± 0.40
Phenolic Acid	1.42 ± 0.04	1.64 ± 0.05	1.60 ± 0.12	1.65 ± 0.03	2.50 ± 0.06	2.18 ± 0.05	1.54 ± 0.01	1.40 ± 0.01
Superoxide	0.03 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Proline	0.09 ± 0.01	0.12±0.00	0.08 ± 0.00	0.10 ± 0.01	0.12 ± 0.01	0.09 ± 0.00	0.06 ± 0.01	0.07 ± 0.01
Unknown	81.15 ± 1.14	76.76 ± 2.34	80.55 ± 1.87	77.12 ± 1.63	73.91 ± 1.25	70.75 ± 0.58	80.42 ± 0.76	71.70 ± 2.95

4.3 DISCUSSION

4.3.1 Some physiological responses of *in vitro E. grandis* shoots to the different non-freezing low temperature and culture periods

The physiological characteristics of the *in vitro E. grandis* shoots were not negatively impacted by the non-freezing low temperature and culture period (chilling) treatments. The viabilities remained 100%, while the WC, emergence of axillary buds, and tissue browning scores were similar between treatments and with the controls (Figs 26 and 27, and Tables 9 and 10). The lack of impact by these conditions provided a positive indication that these chilling treatments had not stressed the material (i.e. no drastic changes occurred at a physiological level). This indicated that underlying resistance mechanisms allowed for tolerance to these conditions of strain. However, based on the morphological observations (Table 11), it appeared that growth may have been reduced by chilling. These physiological responses were similarly reflected with clones of *E. globulus*, which were shown to have reduced growth in response to chilling (Shvaleva *et al.*, 2008). Water status also remained unaffected by chilling in *E. globulus* clones and from other plant species (Equiza *et al.*, 2001; Shvaleva *et al.*, 2008); similar to the present observations with *in vitro E. grandis* shoots.

However, there were instances of subtle changes to certain physiological characteristics that occurred during growth under normal growth room conditions, following the chilling treatments. Growth of shoots (vigour) was promoted under growth room conditions and emergence of axillary buds was more evident, after treatment of shoots at 10°C for 3 days (Figs 27 and 28). The formation of callus was not as high as the controls after the shoots were treated at 10°C and 15°C for 3 days (Table 10). It would seem that the treatment of 10°C for 3 days had induced underlying adaptive metabolism that protected the tissues of shoots and axillary buds during the chilling treatment. Similar underlying responses were also seen in shoots that were treated at 15°C for 3 days, even though this temperature fell within the preferred temperature range for growth but, because of the decreasing temperature trend, responses were triggered. Adaptive metabolism may have ensured that when favourable conditions returned, the transition to growth was less likely to be impeded by the effects of chilling. This type of response could be related to a seasonal response, since 10°C falls below the preferred *E. grandis* temperature range for growth. This could represent the transition usually associated with seasonal changes (from autumn and winter to spring and summer), thereby resembling the overwintering process (Wisnieski *et al.*, 2003). Such a seasonal

influence was demonstrated with *in vitro* axillary buds of mulberry, where during artificially simulated summer conditions there were higher sprouting frequencies and development of axillary buds into shoots than during winter stimulated conditions (Vijaya Chitra & Padmaja, 2002).

Although the above mentioned physiological characteristics showed little or no changes, morphological observations suggested that photosynthesis was affected by these low temperature treatments (Table 11). Unpublished observations from our laboratory have shown that *in vitro* shoots cultured for more than 3 days at 4°C displayed the same visible symptoms of yellow and browning of leaves. Similarly, Yadav (2010) also reported similar findings and suggested that yellowing and browning of leaves was an associated effect of low temperature. Under these treatments, photosynthesis is slowed, and if the duration and intensity is prolonged beyond tolerable capacity it can reduce productivity and negatively impact survival as indicated by signs of senescence (Chalker-Scott, 1999; Kratsch & Wise, 2000; Steyn *et al.*, 2002). When photosynthesis is slowed, the harvesting of light energy during the photoperiod does not stop, and because of this energy capture continues at a fast rate, while photosynthetic electron transport and dissipation is drastically slowed (Chalker-Scott, 1999; Steyn *et al.*, 2002).

This occurrence results in photoinhibition, where the over-absorbance of light energy during slowing photosynthetic capacity represses overall photosynthesis (Chalker-Scott, 1999; Close *et al.*, 2000; Steyn *et al.*, 2002). This can lead to the formation of ROS which, in turn, causes disruptions in primary metabolism, photodynamic bleaching, and ultimately reduced growth and development (Chalker-Scott, 1999; Steyn *et al.*, 2002). In order to attenuate the impact of photoinhibition, anthocyanins and carotenoids are accumulated in leaves and peripheral tissues (Chalker-Scott, 1999; Close *et al.*, 2000; Steyn *et al.*, 2002). This is especially the case with anthocyanins, which are transiently accumulated under unfavourable conditions, and thus when favourable growth conditions returns are degraded (Chalker-Scott, 1999; Close *et al.*, 2000; Steyn *et al.*, 2002). Such accumulations are visible as reddish pigmentations (Chalker-Scott, 1999; Close *et al.*, 2000; Steyn *et al.*, 2002), very much like that observed with *E. grandis* shoots that were chilled in this study (Table 11). Accumulation of these red pigments, especially anthocynanins, are metabolically costly to produce and thus are locally accumulated in epidermal and mesophyll layers of leaves and around the vascular parenchyma of the mid-rib, as well as the stem that are directly exposed to light (Chalker-Scott, 1999; Steyn *et al.*, 2002;). Anthocyanins and carotenoids can function as light screens,

which attenuate the amount of light that is absorbed (Chalker-Scott, 1999; Close *et al.*, 2000; Steyn *et al.*, 2002). Along with the accumulation of these pigments, it has also been proposed that low levels of chlorophyll may passively reduce the absorption of excess irradiance (Close *et al.*, 2000), hence the visible symptoms of light green leaves in shoots in this study (Table 11). Cold hardened seedlings of *E. nitens*, for example, have been shown to have lowered photosynthetic activities and chlorophyll content, along with increased accumulation of carotenoids and transient anthocyanins (Close *et al.*, 2000). The same reduction in chlorophyll content was also seen with the sensitive *E. globulus* clone ST51 after chilling (Shvaleva *et al.*, 2008). Thus, *E. grandis* shoots that were exposed to non-freezing low temperatures may have transiently accumulated anthocyanins and decreased chlorophyll pigments as a means of tolerance until favourable conditions returned.

4.3.2 Biochemical responses of shoots to the different non-freezing low temperature and culture periods

Although the effects of the non-freezing low temperatures and culture periods were limited at the physiological level, the biochemical data showed that the conditions had influenced some biochemical responses within the *in vitro E. grandis* shoots. These responses were noted as changes in levels of abiotic stress-related biomolecules, and the changes suggested that the pattern of biochemical responses induced were associated with specific chilling conditions.

4.3.2.1 Starch and soluble carbohydrate responses

The non-freezing low temperatures and culture period did not induce changes to starch and TSS content within *in vitro E. grandis* shoots, since both levels remained statistically similar to the controls (Figs 29 and 30). As such, the results show the flexibility of the starch- and carbohydrate-related metabolic activities of the *E. grandis* to the non-freezing low temperatures. It was also possible that the culture periods may not have been long enough to induce significant changes in the starch and TSS levels. As gleaned from literature, culture periods for cold acclimation are generally longer than 2 days and indeed may last for several weeks, before significant responses in starch and carbohydrate levels are observed (Burbulis *et al.*, 2011; Costa E Silva *et al.*, 2008; Jouve *et al.*, 1993; Morsy *et al.*, 2007; Palonen *et al.*, 2000; Paul *et al.* 1991; Rosa *et al.*, 2009; Shvaleva *et al.*, 2008; Travert *et al.*, 1997; Vagujfalvi *et al.*, 1999).

The marginal starch and TSS fluctuations recorded may have occurred within a primary metabolic range, where the starch and carbohydrate metabolic transitions may have indicated low temperature-dependent thresholds in primary metabolism. These chilling-dependent fluctuations in shoots may be especially apparent after the 3 day culture; where the 5°C treatment may have caused a metabolic transition associated with the 'minimum threshold' of primary metabolism (Figs 29, 30 and 34). On the other hand, the 10°C and 15°C treatments may be associated with the transition to the 'maximum threshold' (Figs 29, 30 and 34), when temperatures were sub-optimal from the optimal range of 14°C and 25°C (Booth & Pryor, 1991).

Although there was no significant difference in starch and TSS levels (Figs 29, 30 and 34), it is speculated that the observed fluctuations may indicate starch was converted into soluble sugars in order to compensate the necessary levels of carbohydrates for energy- and tolerance-related functions. In this regard, the rate of photosynthesis would have been reduced on account of continued culture at 5°C. Starch-sugar conversions are a natural response of plant species encountering seasonal changes (Allen & Ort, 2001; Kaplan *et al.*, 2006). Starch content conversions have been noted to occur with the onset of low temperatures during late autumn as photosynthesis is disrupted, and thus seen as a characteristic of cold acclimation and chilling for temperatures between the range of 0°C and 12°C (Allen & Ort, 2001; Kaplan *et al.*, 2006). For example, cold acclimation was noted to have also increased accumulation of water soluble carbohydrates in *T. aestivum* seedlings, *in vitro* rapeseed shoots, and raspberry plantlets (Burbulis *et al.*, 2011; Palonen *et al.*, 2000; Vagujfalvi *et al.*, 1999). These starch-sugar conversions could be the result of vacuole- and plastid-located β-amylase, which is involved in the hydrolytic breakdown of transitory and storage starch that are further metabolised into reducing sugars (Kaplan, 2007; Kaplan *et al.*, 2006).

Furthermore, it is also speculated that the observed fluctuations after 3 days culture at 10°C and 15°C promoted elevated starch and carbohydrate metabolic activities (Figs 29, 30 and 34). Starch may have been converted into soluble sugars, and concomitantly energy-related carbohydrates might have been synthesised during slowing photosynthesis, to compensate for the decreasing starch levels. At the same time, the production of chilling-related photoprotective carbohydrates may have been initiated in addition to the synthesis of photosynthetic carbohydrates. Accumulation of soluble carbohydrates after similar chilling treatments were also reflected in tropical *Coffea canephora* microcuttings (Jouve *et al.*, 1993)

and cold-sensitive *Oryza sativa* seedlings (Morsy *et al.*, 2007), specifically that of sucrose. Similar to the chilling treatments of this study, the described starch and soluble carbohydrate trend was also noted in cotyledons of *Chenopodium quinoa* (Rosa *et al.*, 2009), *in vivo* raspberry cultivars (Palonen *et al.*, 2000), and *Helianthus annuus* seedlings (Paul *et al.* 1991).

The carbohydrate responses for *in vitro E. grandis* shoots were also reflected in other *Eucalyptus* species. Cold-sensitive *E. cypellocarpa* × *E. globulus* cell-suspension cultures showed a slight increase of total soluble carbohydrate after 5 days of cold acclimation at 16°C, but these levels remained similar to the control cultures at 25°C (Travert *et al.*, 1997). Cold-sensitive clone ST51 of *E. globulus* also showed higher accumulation of soluble sugars after cold acclimation at 10°C/6°C (day/night) for 5 days, whereas after only 1 day soluble sugar concentration remained comparable with the control treatment at 24°C/16°C (day/night) (Costa E Silva *et al.*, 2008). The same clone chilled at 10°C/5°C (day/night) for 1 day or 7 days also showed higher accumulation of several individual carbohydrates, compared with the controls, where sucrose and glucose were the most dominant (Shvaleva *et al.*, 2008). The culturing of the ST51 clone for 7 days at 10°C/5°C (day/night) increased accumulation far greater than at 1 day, which correlated with decreased growth and the slowdown of photosynthesis (Shvaleva *et al.*, 2008). Thus, Shvaleva *et al.* (2008) suggested that the increased soluble carbohydrates after chilling (10°C/5°C) reflected a reduction in sink strength (i.e. starch) of the aboveground plant tissues. However, the metabolic transition of *in vitro E. grandis* shoots at 10°C and 15°C showed higher starch accumulations than the controls, and only with the 5°C treatment did starch content decline and the soluble sugars slightly increase (Figs 29 and 30).

This transitory metabolism in starch and carbohydrates could also have arisen as a result of temperature-dependent circadian regulation (Epinoza *et al.*, 2010; Mas & Yanovsky, 2009). Circadian regulation of starch-carbohydrate partitioning by diurnal rhythm has been well documented with the model-plant *A. thaliana* (Epinoza *et al.*, 2010; Lu *et al.*, 2005), and even in the leaves of *Beta vulgaris* L. (Li *et al.*, 1992). Under the diurnal rhythm, starch synthesis occurs at a steady rate during the day cycle (warmer temperatures), and before the transition into the night cycle (lower temperatures) there is an elevated accumulation of transitory starch in chloroplasts (Epinoza *et al.*, 2010; Li *et al.*, 1992; Lu *et al.*, 2005). The transitory starch is then regulated in its degradation during the night cycle as a sink source for energy-related carbohydrates for respiration, when there is a slowdown of photosynthesis that is leading into its suppressed activity during the night cycle (Epinoza *et al.*, 2010; Li *et al.*, 1992;

Lu *et al.*, 2005). Despite a reduced photosynthetic capacity because of the *in vitro* conditions supplying carbohydrates to the plant tissues (see Chapter 1), the chilling treatments may have further impacted the reduced photosynthetic capacity of the *in vitro E. grandis* shoots which may have been then compensated for by circadian regulation. This may have resulted in the transitory synthesis of starch and carbohydrates after 3 days culture at 10°C and 15°C, and when temperature decreased to 5°C, circadian regulation may again have compensated for this change leading to the directing of responses towards the initiation of cold tolerance. However, this would have to be experimentally verified for the *in vitro E. grandis* shoots, since starch and soluble carbohydrates in this study were simultaneously quantified around the same time of the day for all treatments.

4.3.2.2 Proline responses

Under growth-promoting conditions, accumulation of proline plays an important role during growth and development of *in vitro E. grandis* shoots, and this was only evident after 3 days of culture on acclimation medium where its synthesis was presumably increased for the purpose of growth and development (Fig 31; and see Section 3.3.2). When shoots were treated at 5°C for 1 day, 10°C and 15°C, proline metabolism appeared to be unchanged by these treatments, which resulted in levels that remained similar to the 1-day control (Fig 34). This similarity between proline content was also evident between the control (25°C) and after cold acclimation at 12°C for 12h of *in vitro E. grandis* shoots (Souza *et al.*, 2004). In contrast, the *E. globulus* clone ST51 showed that after cold acclimation at 10°C/5°C (day/night) for 1 day proline levels reduced and it further decreased after 7 days, when compared with the control (25°C/16°C) that had consistently high proline levels (Shvaleva *et al.*, 2008).

When shoots were cultured for 3 days at 5°C, the proline level extracted from shoots under this condition was maintained at a level as high as the level from the 3-day control (Fig 31), whereas the other chilling temperatures resulted in lower levels of proline. This proline level in shoots after treatment at 5°C for 3 days indicated a response trigger at this temperature (Fig 31). A lower temperature trigger was similarly reflected in the study by Souza *et al.* (2004), when *in vitro E. grandis* shoots were exposed to 0°C for 1h or 3h. However, in that study, accumulation of proline was far greater than the control. A longer culture period trigger was also evident with the *E. globulus* clone ST51, which showed that only after 42 days of culture at 10°C/5°C (day/night) did proline increase to levels similar to that of the controls (Shvaleva *et al.*, 2008). This suggested that culturing at 5°C for 3 days may have

triggered accumulation of proline by promoting anabolic activities from both the glutamate and ornithine pathways (Lehmann *et al.*, 2010; Trovato *et al.*, 2008), which are believed to contribute to accumulation under stress conditions in addition to their developmental roles (Trovato *et al.*, 2008). Based on the model-plant *Arabidopsis*, the glutamate pathway is linked to the cytosol and chloroplast, whereas the ornithine pathway is specific to the mitochondrion (Lehmann *et al.*, 2010; Trovato *et al.*, 2008). Thus, proline accumulation would be immediate and localised to each organelle and the cytosol, in order to compensate for the limited or inhibited activities of development and importantly tolerance.

Although proline itself has been implicated in low temperature tolerance, it may also be possible that the lowered proline levels under these low temperatures were utilized in the synthesis of cold-related proteins and even chaperones (Reierson & Rees, 2001; Trovato *et al.*, 2008). However, Makwarela (1996) showed that there were no cold-related proteins synthesised from *in vitro E. grandis* material that was exposed for 12h at 4°C, other than those involved in normal function. Similarly the results of Souza *et al.* (2004) showed that there were no qualitative differences in protein profiles between *in vitro E. grandis* shoots of the cold treatments and the control.

4.3.2.3 Phenolic acid and superoxide responses

Phenolic acid level increased in the control *in vitro E. grandis* shoots that were cultured at growth room conditions (compare 1 day with 3 days, Fig 32). This suggested that the longer culture period had promoted phenolic acid accumulation to compensate for the increased oxidants (superoxide) produced as a result of some photosynthesis and high respiration activities during developmental programmes of growth (see Section 3.3.2). Thus, superoxide levels were minimized to tolerable levels similar to that of the 1-day control (Figs 33 and 34).

The non-freezing low temperatures and culture period also induced significant changes to the phenolic acid and superoxide content within *in vitro E. grandis* shoots. Treatment of shoots at 5°C, 10°C and 15°C promoted phenolic acid synthesis (Fig 34), with the 10°C and 15°C treatments superoxide levels remaining similar to the controls (Figs 32 and 33), whereas shoots treated at 10°C for 3 days accumulated more phenolic acid (Fig 32). Similar responses of shoots after treatment at 10°C and 15°C indicated that these conditions were tolerable for *in vitro E. grandis* (Fig 34); this was also similarly observed with other non-woody plant species treated at similar temperature ranges. Germinated watermelon and tomato plants, for example, showed that after treatment at 15°C total phenols had increased (Rivero *et al.*, 2001).

Phenolic content of androgenic Festulolium genotype 621 (Pociecha *et al.*, 2009) and *in vitro Miscanthus giganteus* rhizomes (Plazek *et al.*, 2011) also showed high accumulation after pre-hardening at 12°C and a further increase after hardening at 5°C. Low temperature stress is known to promote phenolic acid production and these may be incorporated into the cell wall, where the adhesion of the cell membrane to the cell wall is promoted and water status of tissues are maintained during low temperature stress (Solecka, 1997). In addition, the high phenolic acid in shoots could be precursors for the synthesis of anthocyanins (see Section 4.3.1), where maximal pigmentation in plants is suggested to occur approximately around 10°C (Solecka, 1997; Steyn *et al.*, 2002).

However, *E. grandis* shoots exposed to 5°C for 3 days had high levels of superoxide that could not be ameliorated by the phenolic acid (Figs 32, 33 and 34). Thus, the treatment of 5°C for 3 days may have become straining for shoots to tolerate. This suggested that the temperature-sensitive and high-energy electron transport chains of metabolism were impacted, as there was an overproduction of superoxide (Foyer & Noctor, 2005; Gill & Tuteja, 2010). At the same time, the suppression of photosynthesis may have lead to the phenomena of photoinhibition (see Section 4.3.1).

4.3.2.4 Indications from the contribution of biomolecules to the fresh weight of shoots (FWS)

Although there were substantial changes in the levels of each biomolecule under these chilling treatments, the contribution of these biomolecules in the context of the *in vitro E. grandis* FWS differed. Of the biomolecules, TSS contributed the highest proportion to the FWS, and this was followed by starch and phenolic acid (Table 12). Starch and TSS are important biomolecules, involved in photosynthesis and respiration (see Section 4.3.2.1), whereas phenolic acid are potent antioxidants that are involved in minimizing oxidants (dangerous by-products of metabolism) as well as pre-cursors of cell wall components (see Sections 1.5.2.2 and 3.2.2). However, these three biomolecules combined were not the greatest contributors to the FWS.

Unknown components were the highest of the proportions in the FWS, which decreased in proportion after treatment to various experimental conditions (Table 12). When the unknown component proportions decreased, there were increased gains in the TSS proportions across the different conditions, where the 10°C and 15°C treatments resulted in highest gains (Table 12). This proportional re-allocation suggested that there were metabolic shifts induced under these conditions that triggered increased soluble sugar accumulation and subsequent

conversion into starch. Hjelm and Ogren (2003) reported a similar trend in their investigations of three tree species *Phalaris*, *Salix* and *Picea*. Low temperatures induced surpluses of carbohydrates in these species, which were effectively converted into storage polysaccharides such as starch and fructan while growth rate (cell expansion and shoot elongation) was depressed (Hjelm & Ogren, 2003). It is believed that elevated levels of carbohydrates can suppress photosynthesis under low temperatures, and thus trees strategically respond by converting the surplus into a storage-sink source, which permits photosynthesis to operate independently of growth, since growth varies with temperatures (Ensminger *et al.*, 2006; Hjelm & Ogren, 2003).

Despite the commonality of biomolecule contribution under the various experimental conditions, treatment of shoots at 10°C had the strongest impact on their contributions to the fresh weight of shoots. The proportions of the TSS, starch and phenolic acid were amongst the highest, while the unknown components were the lowest under this condition (Table 12). This clearly suggested that 10°C induced biochemical responses involving metabolic shifts and re-allocations within the FWS, especially noticeable after 3 days of culture. Proportions for starch and unknown components decreased after 3 days of culture, which may have contributed to the increased TSS proportions (Table 12). Phenolic acid proportions, on the other hand, reduced after 3 days of culture, since phenolic acid may have been was utilized in minimizing superoxide levels and incorporated into cell walls (Table 12).

4.4 SUMMARY AND CONCLUSION

All the non-freezing low temperatures and culture periods, and even control conditions, had some influence on the physiology and biochemical processes of *in vitro E. grandis* shoots. In particular, the shoot physiology suggested that photosynthetic activities were slowed down. Biochemical responses included the increased synthesis of abiotic stress-related biomolecules or maintained them at control levels. However, there is strong evidence from the data that the abiotic stress-related biochemical pathways may be governed by specific condition ranges and biochemical changes. These conditions and changes include prolonged culture of shoots within a specific non-freezing low temperature range and perhaps overproduction of oxidants (superoxide) to promote synthesis of abiotic stress-related biomolecules (Fig 34). All of which may be induced by upstream molecular responses that may have been activated by signalling pathways originating from organelle-based sensors (e.g. chloroplasts and mitochondria).

In vitro shoots treated under the control condition (25°C) for 3 days promoted primary metabolic activities as reflected in the increases in proline and phenolic acid production for growth and development (Fig 34). Treatment of shoots at 15°C promoted starch and carbohydrate metabolic fluctuations (Fig 34), promoted phenolic acid and anthocyanin accumulation (Fig 34; and see Section 4.3.1), and maintained 1-day control proline levels (Fig 34), all of which may have contributed in minimizing superoxide levels to tolerable control levels (Fig 34). The condition of 10°C for 3 days specifically triggered increased accumulation of phenolic acid and anthocyanins (Fig 34; and see Section 4.3.1), while other biomolecule changes were similar to the responses under 15°C. The culturing of shoots for 3 days at 5°C caused the greatest strain, which may have escalated the rate of superoxide overproduction (Fig 31). This overproduction of superoxide could not be alleviated to control levels by the higher proline levels (Fig 34) nor the increased phenolic acid levels (Fig 34), and starch and carbohydrate metabolic adjustments (Fig 34). However, regardless of the treatments, the contribution of each biomolecule to the FWS indicated that carbohydrate, starch and phenolic acid were the dominant biomolecules involved in tolerance and that there were also unknown components responding (Table 12).

In light of the responses of the shoots and in line with the purpose of the study, it was thus inferred that the chilling treatment of 10°C for 3 days would be the appropriate 'cross-acclimation' condition for *in vitro E. grandis* shoots in preparation for subsequent physical drying. This inference was based on the positive biochemical responses under this condition, further supported by the downstream physiological responses of shoots, where growth of shoots (vigour) was promoted and callus formation was minimized after returning to growth room conditions (Figs 27 and 28, and Table 10).

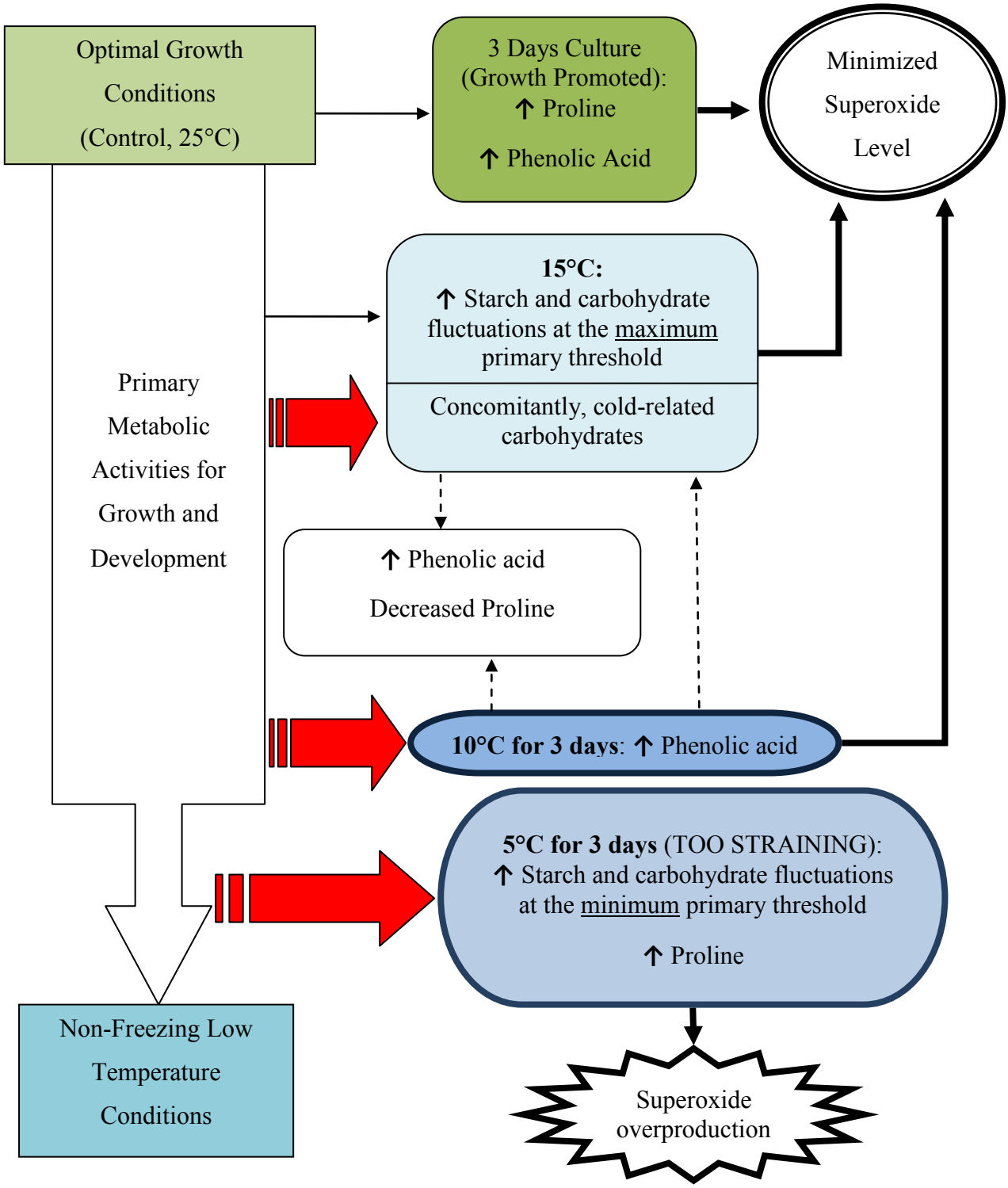


Fig 34. Schematic representation of the specific biochemical responses from *in vitro E. grandis* shoots after being treated different cold treatment conditions. Thin horizontal arrows indicate metabolic fluctuations during primary metabolic activities due to cold conditions. Red arrows indicate cold-condition specificity of biochemical responses, inducing additional or alternative pathways. Thick black arrows indicate the biochemical effect of these biomolecule changes. Dashed arrows indicate complementary responses (part of primary metabolic activities) to the main biochemical response. (↑): increased, and (↓): inducing another response.

CHAPTER 5.

Understanding some of the physiological and biochemical responses of *in vitro Eucalyptus grandis* shoots to physical drying

5.1 INTRODUCTION AND LITERATURE REVIEW

In literature, the physiological and, to a lesser extent, biochemical responses of *Eucalyptus* species to dehydration stress appear to be determined mainly from trees in the field, and seedlings or micropropagated plantlets in greenhouses or growth chambers (Table 13). However, the responses of *in vitro* cultures of *Eucalyptus* species to dehydration stress, especially that of *E. grandis*, appear to be limited to physiological responses (Table 13). In the context of the cryopreservation of *in vitro E. grandis* tissue (see Sections 1.3 and 3.1), it is therefore no surprise that physiological factors (e.g. water content, viability and vigour) are integral measurements for primarily evaluating the responses to dehydration stress, whereas biochemical responses have received far less attention (Table 13). Nonetheless, there is literature available about the physiological and biochemical responses of *Eucalyptus* species to dehydration stress but the responses are specific to the type of material and form of dehydration (Table 13). For example, the physiological and biochemical responses assessed for *Eucalyptus* seedlings and micropropagated rooted-cuttings exposed to artificially imposed drought, or even trees in the field that encounter natural seasonal occurrences of drought (Table 13). Despite these inherent differences, the literature does provide some perspective on the physiological and biochemical responses of *Eucalyptus* species to dehydration stress.

In the study by Padayachee *et al.* (2008), *in vitro E. grandis* axillary buds were selected as cryopropagules and physical drying was considered an appropriate pre-treatment approach for cryopreservation. The study showed that after the first 20 min of drying the water content of axillary buds decreased from 0.73g water/g FWS (control) to 0.37±0.01g water/g FWS, but this was also accompanied by a substantial loss in viability from 90.00±5.00% to 41.66±1.66% (Padayachee *et al.*, 2008). As physical drying time was increased to 40 min and 60 min, water content further reduced to 0.31g water/g FWS and 0.19g water/g FWS respectively, but viability had also sharply declined to 10.0±3.0% and 0.0% respectively (Padayachee *et al.*, 2008). In response to the physical drying, the axillary buds displayed varying degrees of ultra-structural changes (Padayachee *et al.*, 2008). These were classified as moderate through to extensive damage, yet there were signs of intact cells, especially after

20 min of drying (Padayachee *et al.*, 2008). It was therefore concluded by those authors that for the cryopreservation of axillary buds, a 20 min pre-treatment by physical drying would be appropriate (Padayachee *et al.*, 2008). That particular study therefore provided some perspective on the physiological responses of *E. grandis* to dehydration stress. However, the study was specific to isolated axillary buds, which are not as developmentally complex and as large as the shoots used in the present study. In addition, that study did not elaborate on the physiological and biochemical responses that imparted tolerance, which ultimately ensured the viability of some axillary buds.

Other studies on biochemical and physiological responses of *Eucalyptus* species to dehydration stress have also provided some additional insight to their responses that imparted tolerance (Table 13). Seedlings of several *Eucalyptus* species physiologically showed declining photosynthetic rate, stomatal conductance, and increased senescence of leaves when exposed to conditions of soil water deficits and droughts (Gindaba *et al.*, 2004; Kirschbaum, 1987; Merchant *et al.*, 2007). Such seedlings were also able to adjust their transpiration capacity according to water availability, and breakdown and re-distribute cellular constituents to regions of new growth, which resulted in reduced overall growth and development (Merchant *et al.*, 2007). The breakdown and re-distribution of cellular constituents (i.e. biochemical responses) also imparted osmotic protection in the seedlings, which was attributed to adaptive metabolism for tolerance that included osmotic adjustments (Callister *et al.*, 2008; Merchant *et al.*, 2007; Shvaleva *et al.*, 2006; Warren *et al.*, 2011ab). This type of tolerance is considered to be induced as an attempt to maintain osmotic potential and ensure cellular protection in cells and tissues, in order to prevent cellular damage and further loss of water (Callister *et al.*, 2008; Merchant *et al.*, 2007; Shvaleva *et al.*, 2006; Warren *et al.*, 2011ab). Such tolerance is strongly attributed to the accumulation of soluble carbohydrates (e.g. sucrose) that plays a predominant role in osmotic adjustments, which along with the active accumulation of amino acids (e.g. proline) and phenylpropanoids (e.g. phenolic acid) provide osmoprotection against dehydration stress and secondary oxidative stress (Callister *et al.*, 2008; Merchant *et al.*, 2007; Shvaleva *et al.*, 2006; Warren *et al.*, 2011ab). Furthermore, the extent of tolerance may also be dependent on plant and leaf age (Chaves *et al.*, 2009; Merchant *et al.*, 2007).

Although these responses to dehydration stress provided some perspective on the physiological consequences and importantly tolerance in *Eucalyptus* species, the type of material and species and the form of dehydration investigated are different from this

particular study. It was therefore necessary to develop an understanding, in the context of cryopreservation, the fundamental biochemical and even physiological responses of *in vitro E. grandis* shoots to an appropriate physical drying period, and the influence that culture period may have on their response to drying.

Table 13. Examples of measured physiological and/or biochemical responses of different material types of various *Eucalyptus* species to dehydration stress

Species	Type of material	Growth condition	Dehydration method	Measured responses		Reference
				Physiological	Biochemical	
E. grandis	Axillary buds	*In vitro* culture	Desiccation over activated silica gel for 20-60 minutes	Vigour and viability assessments, water content, and ultra-structural microscopy		Padayachee *et al.*, 2008
E. grandis	Shoots, axillary buds, and pollen	*In vitro* culture	Drying by using laminar flow for 0-6h	Survival, contamination, and viability and multiplication rates		Thokoane, 1998
E. camaldulensis, *E. grandis*, *E. globulus*, *E. camaldulensis*×*E. grandis*, and *E. camaldulensis*×*E. globulus*	Trees	Field	Seasonal precipitation/evaporation in field site near Wangaratta, Victoria, Australia	*In situ* water potential, and osmotic potential	Carbohydrates, cations and anions, and organic acids	Callister *et al.*, 2008
E. camaldulensis, and *E. globulus*,	One-year-old seedling	Glasshouse	7 days of no watering	Root collar diameter, leaf area, plant height, plant biomass, root/shoot ratio, water loss, water potential and relative water content, and gas exchange		Gindaba *et al.*, 2004
E. cladocalyx	Seedling	Greenhouse	25% of average nutrient solution received by high watered plants	Height, total dry mass, root/shoot ratio, leaf area ratio, leaf weight to area ratio, photosynthesis and stomatal conductance, gas exchange, and transpiration	Cyanogenic glycosides, total phenolic acid, carbon and nitrogen content	Gleadow & Woodrow, 2002
E. pauciflora	Seedling	Glasshouse	Withholding of water	Gas exchange, stomatal conductance, and quantum yield of photosynthesis		Kirschbaum, 1987

Table 13. Continued

E. obliqua, *E. rubida*, *E. camaldulensis*, *E. cladocalyx*, *E.polyanthemos*, and *E. tricarpa*	Five-month-old trees	Greenhouse	20% of average water received by well watered plants	Height, diameter, biomass, leaf water relations, and, photsynthesis and stomatal conductance		Merchant *et al.*, 2007
E. globulus	Rooted-cuttings	Greenhouse	Watering equal to 50% transpiration loss	Water potential, and growth analysis (size and biomass)	Soluble and insoluble sugars, proline and leaf pigments, and antioxidant enzymes	Shvaleva *et al.*, 2006
E. arenacea, *E. delegatensis*, *E. nitens*, *E. regnans*, and *E. socialis*	Seedling	Greenhouse	Reduced irrigation	Water potential, gas exchange, and epidermal conductance	Organic metabolites, and inorganic anions and cations	Warren *et al.*, 2011a
E. dumosa and *E. pauciflora*	Seedling	Greenhouse	2 weeks of slow dry down	Water potential, and leaf water relations	Organic metabolites, and inorganic anions and cations	Warren *et al.*, 2011b

5.2 Determination of the appropriate cryopreparative drying time for *in vitro E. grandis* shoots based on physiological responses

5.2.1 RESULTS

Water content (WC) of *in vitro E. grandis* shoots (inclusive of damaged, and non- and viable tissues and cells of shoots) significantly decreased as drying time increased; the longest tested time of 80 min of drying had the lowest WC (0.28 ± 0.06 g water.g^{-1} FWS) (Fig 35). Statistically, there were no differences in WC after 20, 40 and 60 min of drying.

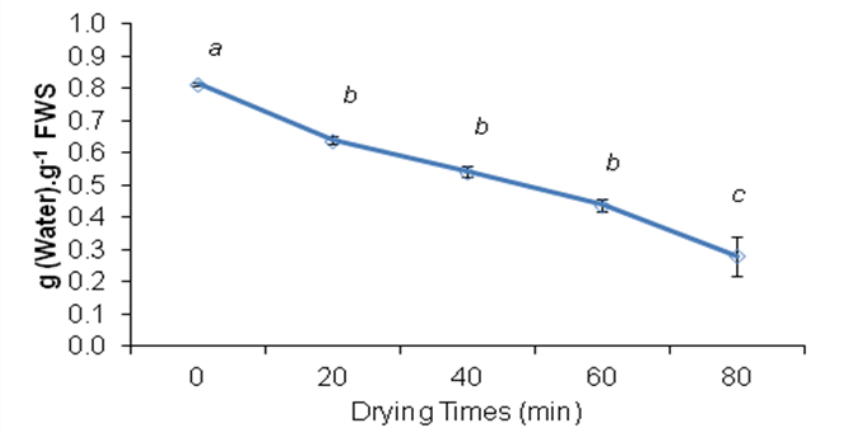


Fig 35. Water content (WC) of *in vitro E. grandis* shoots cultured for 3 days on acclimation medium and subsequently dried over activated silica gel. Open diamond points represent mean ± SE. The letters indicate significant differences (*F(4,10)=56.5, p<0.05*).

Observable changes in the viability were only noted after the second week of evaluation (Table 14). Viability after 20 min and 40 min of drying remained 100% but the viability decreased marginally to 95.6 ± 7.7% after 60 min and dropped significantly to 86.7% after 80 min of drying (Table 14).

Table 14. Viability of *in vitro E. grandis* shoots cultured for 3 days on acclimation medium and then dried over activated silica gel for different drying periods (with standard deviation). Letters indicated that viability significantly decreased after 80 min of drying (*F(4,10)=8.5, p<0.05*).

	Week	0 min	20 min	40 min	60 min	80 min
Viability (%)	0	100	100	100	100	100
	2	100[a]	100 [a]	100 [a]	95.6 ± 7.7[1a]	86.7[1b]

1 Number of viable shoots from a sample of 15: n=14.3±1.2 for 60 min, and n=13 for 80 min

The vigour of the axillary buds (emergence of axillary buds) and shoots (growth of shoots) per explant (vigour rates) were only evaluated for viable shoots (Figs 36 and 37). Vigour significantly decreased as drying time increased (Figs 36 and 37). The lowest rate for the emergence of buds (2.4 ± 0.4 visible axillary buds/week) and growth of shoots (0.7 ± 0.2 visible shoots/week) was observed after 80 min of drying (Figs 36 and 37).

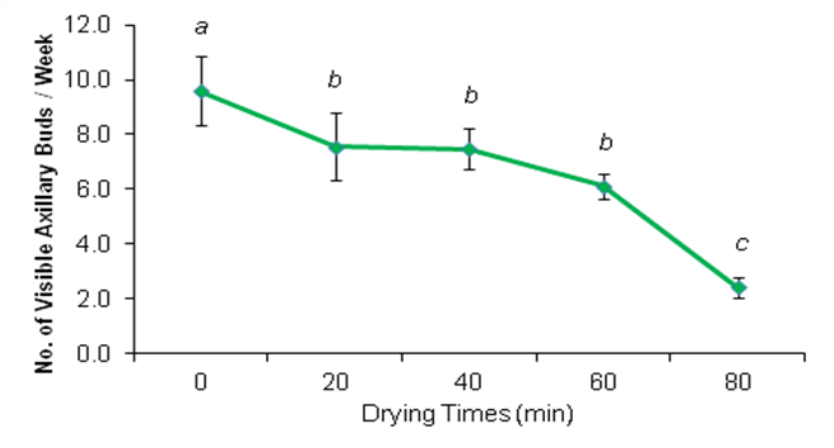


Fig 36. Vigour, in terms of the emergence of axillary buds, of *E. grandis in vitro* shoots cultured for 3 days on acclimation medium and then exposed to different drying times. Diamond points represent mean ± SE. The letters indicate significant (*F(4,10)=10.00, p<0.05*).

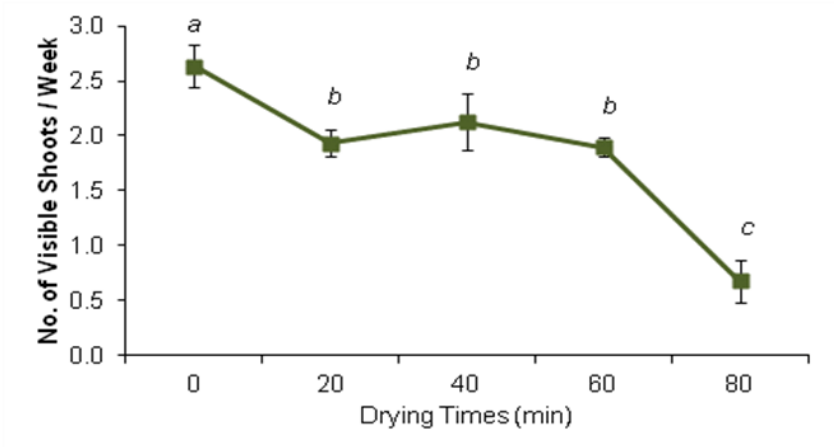


Fig 37. Vigour, in terms of the growth of shoots, of *E. grandis in vitro* shoots cultured for 3 days on acclimation medium and then exposed to different drying times. Square points represent mean ± SE. The letters indicate significant differences (*F(4,10)=22.34, p<0.05*).

Callus formation scores were only evaluated for viable *in vitro E. grandis* shoots (Table 15). Callus formation from non-dried shoots and shoots dried for 20 min was observable after 1 week, while for shoots dried for 40, 60 and 80 min callus was only observed after 2 weeks (Table 15). Assessment of tissue browning (Table 15) showed that scores were the highest at week 1 and by week 2 had decreased or remained the same as week 1, depending on the drying time. Tissue browning score was the highest after 80 min of drying when compared with 0, 20 and 40 min of drying, but there was no difference between the shoots dried for 60 and 80 min (Table 15).

Table 15. Callus formation and tissue browning scores of *in vitro E. grandis* shoots cultured for 3 days on acclimation medium and then dried over activated silica gel for different periods (with SE). There were significant differences in callus formation ($F(4,39)=2.69$, $p<0.05$) and tissue browning scores ($F(4,39)=8.99$, $p<0.05$).

Score (0-5)	Week	0min	20min	40min	60min [1]	80min [1]
Callus Formation	0	0.0	0.0	0.0	0.0	0.1
	1	0.2 ± 0. 2	0.3 ± 0.3	0.0	0.0	0.0
	2	1.0 ± 0.1	1.2 ± 0.2	0.8	0.9 ± 0.1	0.4 ± 0.1
Tissue Browning	0	0.4 ± 0.3	0.7 ± 0.7	0.4	0.5 ± 0.1	0.6 ± 0.2
	1	0.4 ± 0.2	1.2 ± 0.1	2.4 ± 0.2	2.6 ± 0.1	4.1 ± 0.1
	2	1.0 ± 0.1	1.2 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	3.3

1 Number of viable shoots from a sample of 15: n=14.3±1.2, 60 min, and n=13, 80 min

The visible effects of the selected drying times on *in vitro E. grandis* shoots are presented in Fig 38. Although shoots had recovered and grown after 2 weeks on multiplication medium, the occurrence of browning of whole leaves remained more apparent with shoots that were exposed to drying (Fig 38B-E) than in those that were not dried (Fig 38A). The greatest degree of browning was observed in shoots exposed to 80 min of drying (Fig 38E), which correlated with the results of the scores (Table 15). Growth and development of these shoots were also greatly reduced (Fig 38E) compared with shoots exposed to the other drying times (i.e. 20, 40 and 60 min) (Fig 38B-D). In fact, shoots exposed to 20, 40 and 60min of drying appeared to be indistinguishable from each other in their physical appearance (Fig 38B-D), which was also supported by the vigour data (Figs 36 and 37).

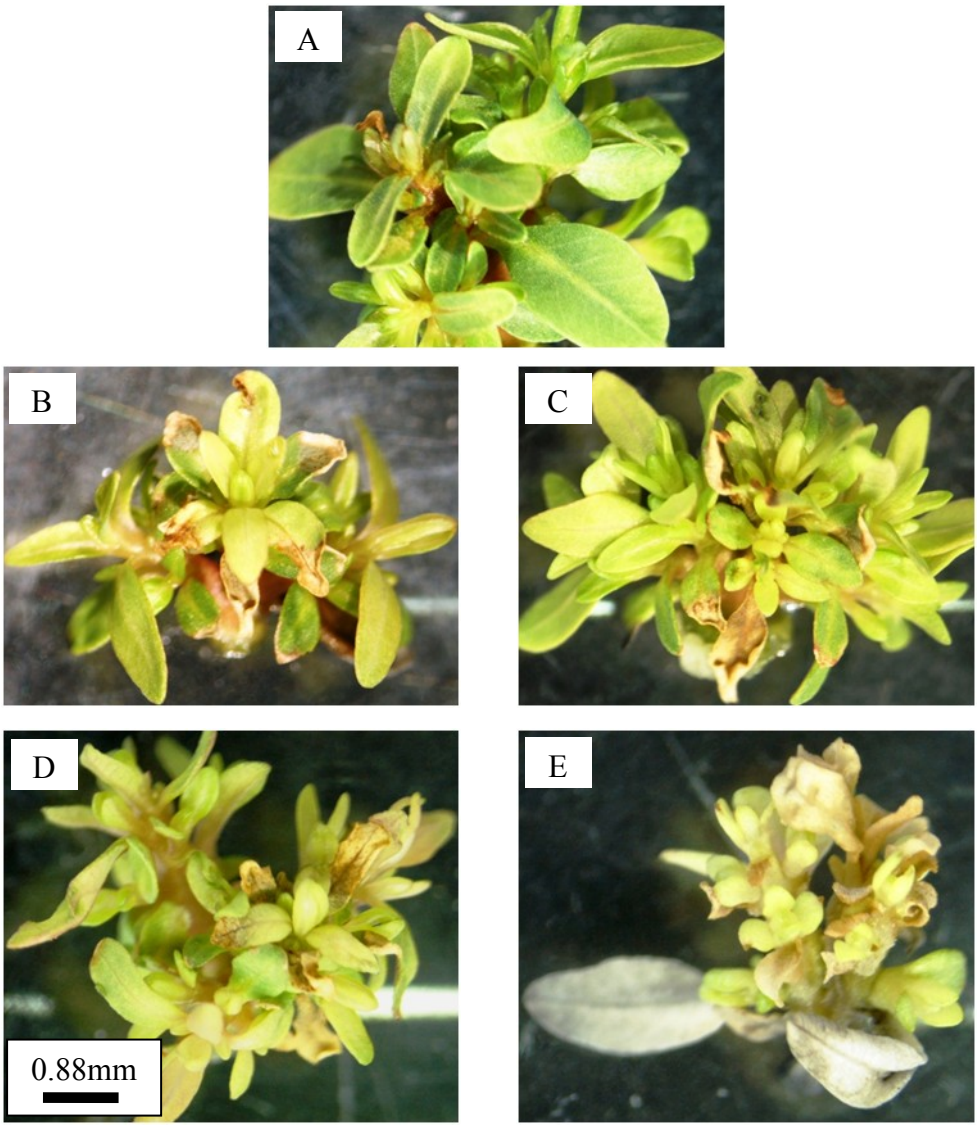


Fig 38. Representative examples of *in vitro E. grandis* shoots (10× magnification) 2 weeks after exposure to no drying (A), 20 min of drying (B), 40 min of drying (C), 60 min of drying (D), and 80 min of drying (E) over 400g of activated silica gel. The representative shoots for (D) and (E) were that of viable shoots which survived the 60 and 80 min of drying.

5.2.2 DISCUSSION

The physical drying of *in vitro E. grandis* shoots for 80 min reduced WC (0.32 ± 0.04 g water.g^{-1} FWS) (Fig 35) to a similar level to that of axillary buds dried for 20 min (Padayachee *et al.*, 2008; and see Section 5.1). The other drying periods (20, 40 and 60 min) also lowered the WCs but these remained statistically similar (Fig 35), yet higher than the WC achieved after 80 min. The statistically similar WCs after 20, 40 and 60 min of drying could be attributed to the activity of underlying biochemical responses ensuring osmotic protection, while 80 min of drying may have partially overcome this protection of osmotic potential (see Section 5.1). However, the longer drying period of 60 and 80 min caused dehydration-related injuries that could not be protected against, hence the reduced viability of shoots by approximately 4% and 13% respectively (Table 14). This was similar to observations in the study by Padayachee *et al.* (2008). Collectively these data confirm that both i*n vitro* axillary buds and shoots of *E. grandis* are sensitive to drying (see Section 5.1). The drying injuries therefore could not be protected against nor prevented by the possible induced stress-related mechanisms of tolerance, irrespective of the vegetative material. However, the more developed and complex shoots may possess mechanisms of tolerance better than the axillary buds (see Section 5.1). This comparison must however be experimentally verified.

The recovery of the dried shoots on multiplication medium confirmed that 80 min of drying had severely reduced growth, and had damaged the shoots. The occurrence of tissue browning, an indicator of tissue and cell death and a symptom of drying stress, was the most widespread on shoots after 80 min of drying, but only noticeable after the first week of recovery growth (Table 15). This suggested that the shoots attempted to repair drying damage, and this was manifested as a lag in the symptom of tissue browning. In some instances the extent of the damage was too severe for recovery purposes, and this may be suggestive of a controlled process of cell and tissue death of sensitive vegetative structures such as leaves. Such a controlled death process would be necessary to prevent further damage of the shoot system. The process of programmed cell death (PCD) could have been triggered by oxidative stress and actions of reactive oxygen species (ROS), and this was manifested as localized browning (Gechev *et al.*, 2006; Lam, 2004; Tiwari *et al.*, 2002; Van Breusegem & Dat, 2006).

Also by the second week, the shoots that had been dried for 80 min had repaired enough to resume growth; this was associated with a reduced spread of tissue browning and the

formation of callus (Table 15). However, the emergence of axillary buds and development into shoots remained slow (Figs 36 and 37). The growth that occurred during recovery from shoots dried for 80 min was mainly of axillary buds (Fig 38), which indicated that axillary bud structures were protected while other tissue structures were sacrificed (e.g. leaves), in order to ensure the survival. Survival of axillary buds may suggest that damages remained within a recoverable threshold, which allowed the inception of the repair mechanism (see Section 1.5.1.1). This recovery may have included osmotic adjustments that ensured osmotic protection during drying, which also permitted the transportation of cellular constituents to axillary buds before sensitive cells and tissues succumbed to the injuries of drying (see Section 5.1).

The other drying periods showed similar vigour responses but not as extreme as from shoots dried for 80 min (Figs 36 and 37). This suggested that these drying times fell within a normal and hence tolerable range which ensured 100% viability. Only after 60 min of drying was tolerance pushed beyond that threshold. In addition, the light-shade of green in recovered shoots, demonstrated the prolonged impacts of drying on photosynthesis, where 80 min of drying had the most severe impact (Fig 38).

The drying period of 80 min was, therefore, selected for the purpose of this study, because the observed WC of the axillary buds after 20 min of drying (Padayachee *et al.*, 2008) was similarly achieved for *in vitro E. grandis* shoots (Fig 35). Furthermore, the impacts on the physiological responses were greater and more definitive after 80 min drying (greater impact on viability with a smaller standard deviation) than with the other drying times (Figs 36-37, and Tables 14 and 15). Thus, the physiological and biochemical responses would be more detectable than from shoots dried for 60 min.

5.3 Some physiological and biochemical responses of *E. grandis* shoots cultured for 3 or 6 days on acclimation medium and subsequently exposed to 80 min of drying

5.3.1 RESULTS

The culture period of 3 or 6 days on acclimation medium were selected according to the results and discussion in Chapter 3 and 4, while the selected cryopreparative drying time of 80 min was based on the results and discussion in Section 5.2. The responses of shoots to culture on acclimation medium for days also represent controls for Chapter 6, where shoots were dried and not dried.

5.3.1.1 Physiological responses

Water content (WC) of *in vitro E. grandis* shoots, cultured for 3 and 6 days on acclimation medium and then dried, significantly decreased when compared with the WC of non-dried shoots (Fig 39). When the dried shoots from both culture periods were recovered on acclimation medium for 24 h, the WCs returned to levels statistically similar to that of non-dried shoots (Fig 39).

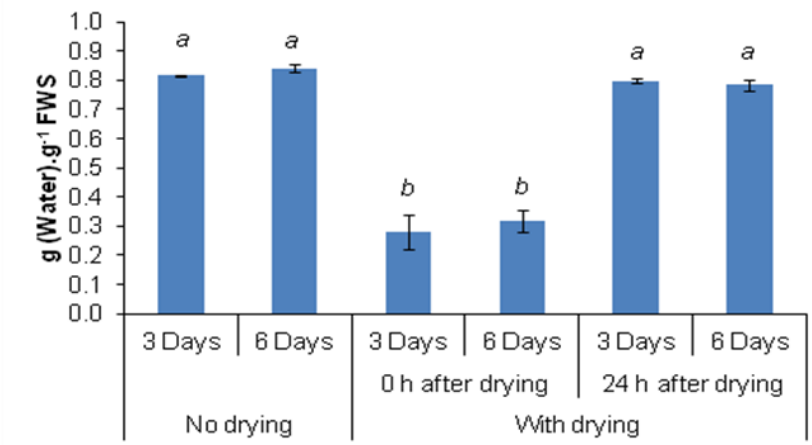


Fig 39. Water content (WC) of *in vitro E. grandis* shoots cultured on acclimation medium for 3 or 6 days and then dried (with SE). Vertical bars represent mean ± SE. WC was established immediately (0 h) and 24 h after drying. The letters indicate significant differences, according to post-hoc Tukey HSD at a 0.05 level of significance (no drying and 0 h: $F(1,8)=0.02$, $p=0.88$; 0 h and 24 h: $F(1,8)=0.44$, $p=0.52$; and no drying and 24 h; $F(1,8)=1.71$, $p=0.23$).

The viabilities of shoots decreased by ±12-14% for both culture periods by the end of the 2 week evaluation period after drying (Table 16).

Table 16. Viability of *in vitro E. grandis* shoots cultured for 3 or 6 days on acclimation medium and then dried (with standard deviation). Drying had significantly reduced viability of shoots, where the reduction was statistically similar between shoots cultured for 3 or 6 days (indicated by letters), according to post-hoc Tukey HSD at a 0.05 level of significance (*F(1,19)=0.14, p=0.72*)

		Without Cryopreparative Drying		With Cryopreparative Drying	
	Week	3 Days	6 Days	3 Days[1]	6 Days[1]
Viability (%)	0	100.00	100.0	100	100
	2	100.00[a]	100.0[a]	86.7[b]	88.9 ± 3.9[b]

1 Evaluated based on the number of viable shoots in a sample of 15: n=13 for 3 Days and n=13.3 ± 0.3 for 6 Days

The vigour in terms of both emergence of axillary buds and growth of shoots from viable shoots, at the end of the 2 week evaluation period, was significantly reduced by drying, irrespective of the culturing periods (Figs 40 and 41). Vigour measured in terms of callus formation scores was significantly lower after drying (Table 17). However, assessment of tissue browning (Table 17) showed that the scores of dried shoots were significantly higher than non-dried shoots. After week 1, tissue browning scores were high and by week 2 had decreased, but remained higher than non-dried shoots (Table 17).

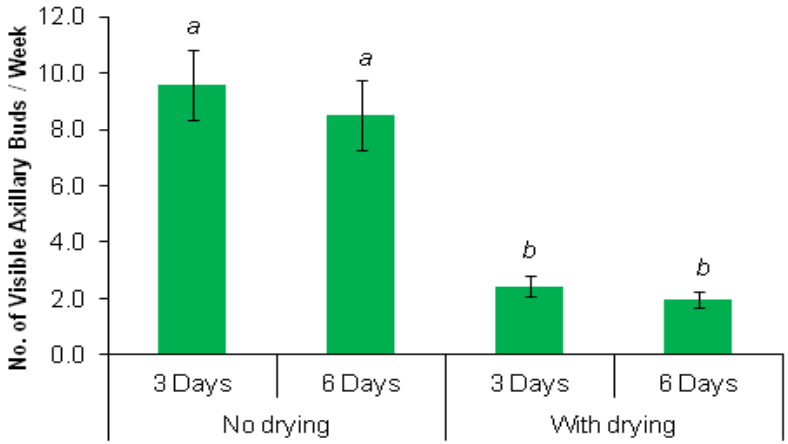


Fig 40. Vigour, in terms of the emergence of axillary buds, of *in vitro E. grandis* shoots cultured for 3 or 6 days on acclimation and then dried. Vertical bars represent mean ± SE. The letters indicate significant differences, according to post-hoc Tukey HSD at 0.05 level of significance (*F(1,8)=0.11, p=0.75*).

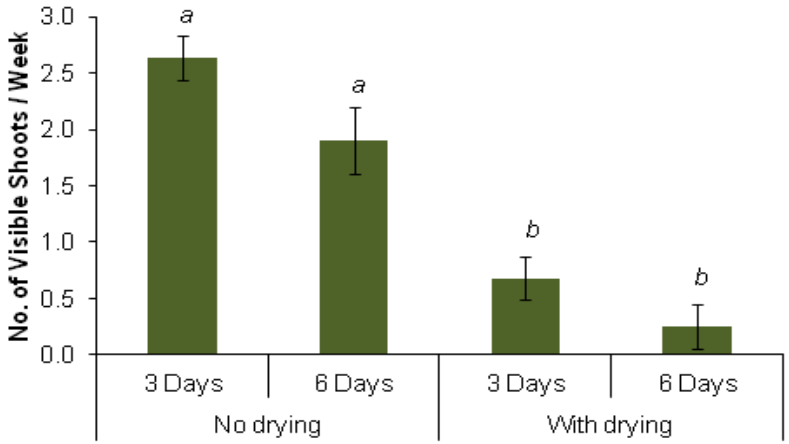


Fig 41. Vigour, in terms of the growth of shoots, of *in vitro E. grandis* shoots cultured for 3 or 6 days on acclimation medium and then dried. Vertical bars represent mean ± SE. The letters indicate significant differences, according to post-hoc Tukey HSD at 0.05 level of significance (*F(1,8)=0.49, p=0.51*).

Table 17. Callus formation and tissue browning scores of *in vitro E. grandis* shoots cultured for 3 or 6 days on acclimation medium without drying and followed by 80 min of cryopreparative drying (with SE). Scores for callus formation were significantly lower for shoots exposed to drying, and tissue browning scores were significantly higher after drying, according to post-hoc Tukey HSD at a 0.05 level of significance (callus: *F(1,31)=1.17, p=0.29*); and tissue browning: *F(1,31)=0.74, p=0.40*). * Evaluated based on the number of viable shoots in a sample of 15: n = 13 for 3 Days and n = 13.3 ± 0.3 (standard deviation) for 6 Days

		Without Cryopreparative Drying		With Cryopreparative Drying	
	Week	3 Days	6 Days	3 Days	6 Days
Callus Score (0-5)	0	0.0	0.2 ± 0.1	0.1*	0.3 ± 0.1*
	1	0.2 ± 0.2	0.8 ± 0.1	0.0*	0.3 ± 0.1*
	2	1.0 ± 0.1	1.2 ± 0.1	0.4 ± 0.1*	0.4 ± 0.1*
Tissue Browning Score (0-5)	0	0.4 ± 0.3	0.8 ± 0.1	0.6 ± 0.2	0.8 ± 0.1
	1	0.4 ± 0.2	1.0 ± 0.0	4.1 ± 0.1	3.5 ± 0.2
	2	1.0 ± 0.1	1.1 ± 0.0	3.3	3.3

Representative examples of dried and non-dried shoots are presented in Fig 42. Non-dried shoots showed small patches of browning on leaves from both culture periods (Figs 42A and D), and clear callus formation on shoots cultured for 6 days (Fig 42D). Immediately after drying, shoots, irrespective of prior culture period, showed wrinkling and folding of leaves with a deep shade of green (Figs 42B and E) and, in some instances, red pigmentation was observed on the stem and the underside of leaves (Fig 42E). After 2 weeks of recovery on multiplication medium, the dried shoots displayed severe and widespread browning of older leaves and tissue structures (Figs 42C and F), and interspersed amongst these were newly developing and developed shoots and axillary buds that were paler green than shoots that had not been dried (Figs 42A and D, and C and F). Close inspection of these recovered shoots showed that smaller developing shoots (4 small developing leaves surrounding the bud) were more common with shoots that had been cultured for 3 days, whereas more developed shoots and axillary buds were associated with shoots that had been cultured for 6 days (Figs 42C and F).

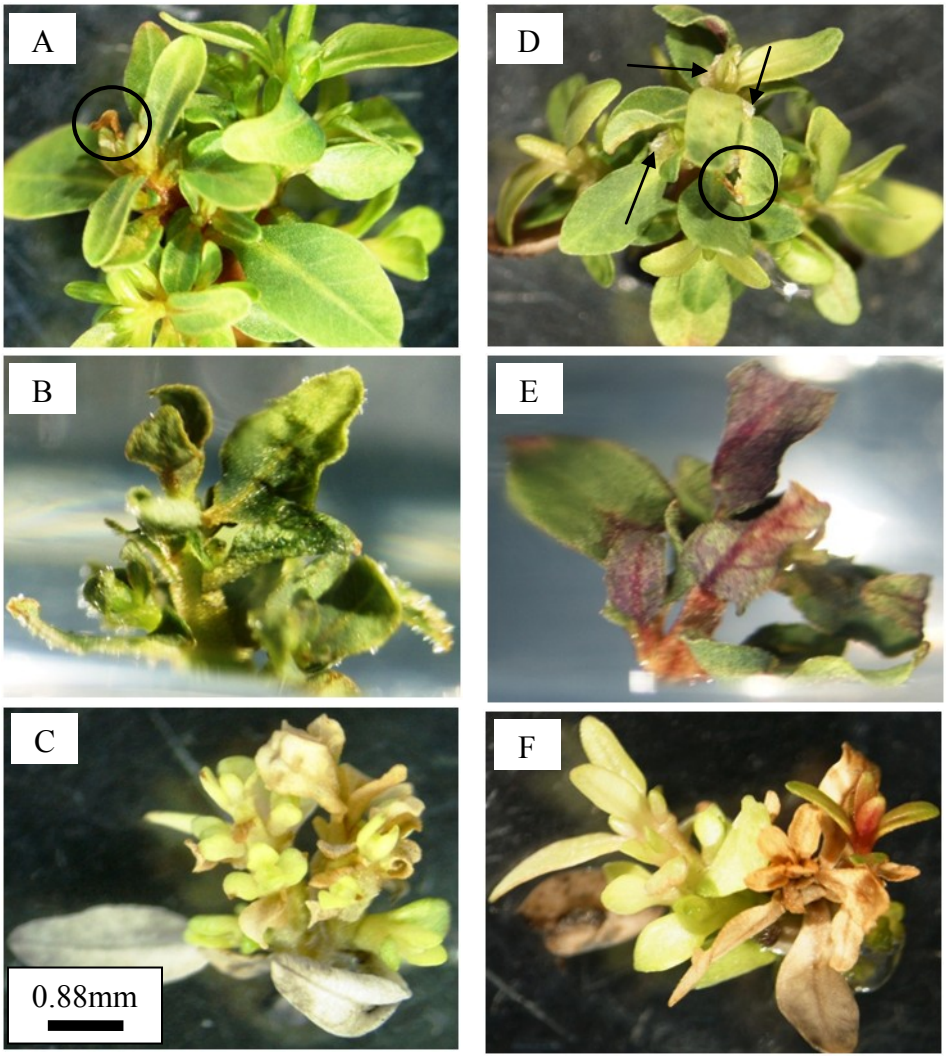


Fig 42. Representative examples of *in vitro E. grandis* shoots (10× magnification), 2 weeks after the following treatments: 1) cultured on acclimation medium for 3 days (A) or 6 days (D); 2) shoots dried for 80 min after 3 days (B) or 6 days (E) culturing on acclimation medium; and 3) after 2 weeks of recovery of dried shoots on multiplication medium that had been cultured post-acclimation for 3 days (C) or 6 days (F) on acclimation medium before drying. The black circle in (A) and (D) indicates the regions with browning of leaves, while the black arrow in (D) indicates the presence of callus.

5.3.1.2 Biochemical responses

5.3.1.2.1 Starch and TSS

The starch levels of the *in vitro E. grandis* shoots cultured on acclimation medium for 3 and 6 days and then dried were lower than those of shoots that were not dried (Fig 43). However, the starch levels were statistically the same between shoots cultured for 3 and 6 days on acclimation medium. In contrast, TSS levels were significantly higher after drying, where the highest level of TSS occurred in dried shoots that were cultured for 3 days on acclimation medium (148.41 ± 5.97 mg GFG.g^{-1} FWS) (Fig 44).

When dried shoots were recovered on acclimation medium for 24h, the respective levels of starch and TSS had significantly reversed, compared with levels observed immediately after drying (Figs 43 and 44). Although TSS levels after 24 h returned to similar levels observed in non-dried shoots, the level for shoots that had been cultured for 3 days (38.09 ± 1.86 mg GFG.g^{-1} FWS) remained significantly high compared with non-dried shoots (Fig 44). Starch levels remained statistically similar between non-dried and recovered shoots, irrespective of culture periods (Fig 43).

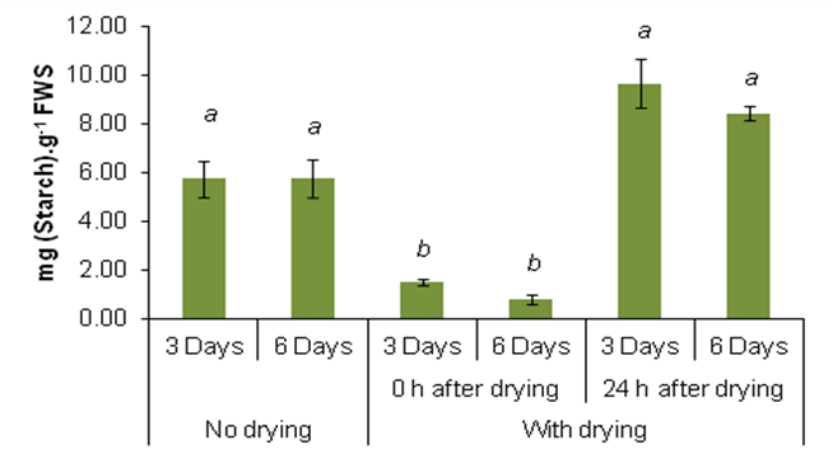


Fig 43. Starch levels of *in vitro E. grandis* shoots cultured on acclimation medium for 3 or 6 days and then dried. Vertical bars represent mean ± SE. Starch levels were established both immediately (0 h) and 24 h after drying. The letters indicate significant differences, according to post-hoc Tukey HSD at a 0.05 level of significance (no drying and 0 h: *F(1,8)=0.44, p=0.52*; 0 h and 24 h: *F(1,8)=0.27, p=0.62*; and no drying and 24 h: *F(1,8)=0.73, p=0.42*).

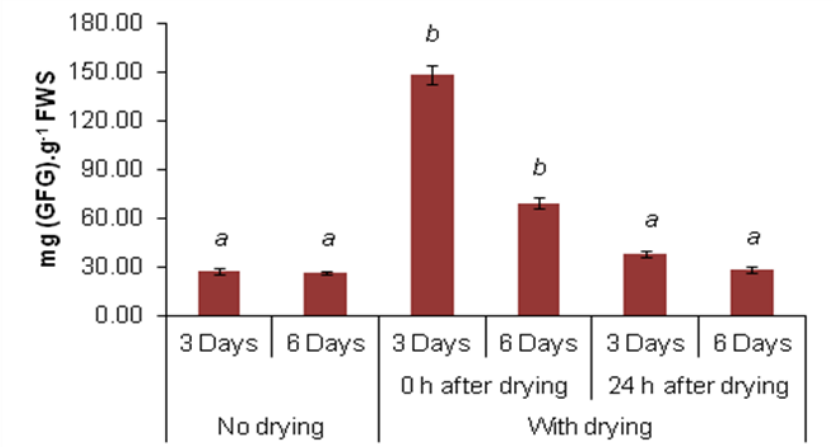


Fig 44. Total soluble sugar, TSS (GFG), levels of *in vitro E. grandis* shoots cultured on acclimation medium for 3 or 6 days and then dried. Vertical bars represent mean ± SE. TSS levels were established both immediately (0 h) and 24 h after drying. The letters indicate significant differences (no drying and 0 h: $F(1,8)=109.16,\ p<0.05$; 0 h and 24 h: $F(1,8)=94.30,\ p<0.05$; and no drying and 24 h: $F(1,8)=6.62,\ p<0.05$).

5.3.1.2.2 Proline

Proline levels extracted from *in vitro E. grandis* shoots that had been cultured on acclimation medium for 3 and 6 days and then dried were significantly higher than the levels from non-dried shoots (Fig 45). Levels from dried and non-dried shoots cultured for 6 days were significantly higher than shoots cultured for 3 days (Fig 45). After the dried shoots were recovered on acclimation medium for 24 h, proline levels decreased, returning to levels statistically similar to that of non-dried shoots (Fig 45).

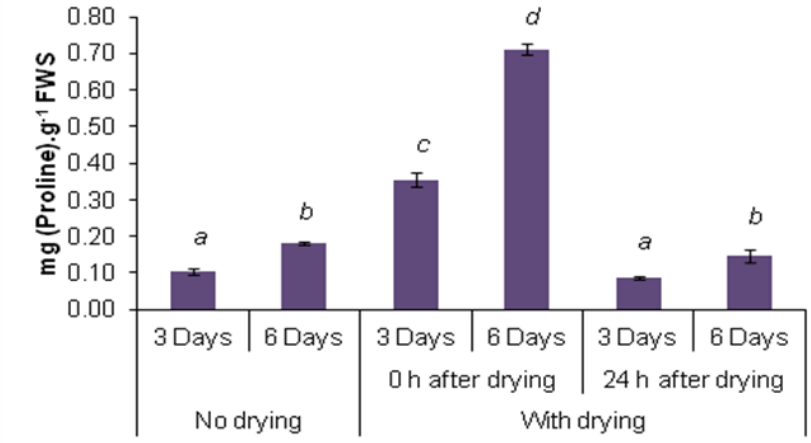


Fig 45. Proline levels of *in vitro E. grandis* shoots cultured on acclimation medium for 3 or 6 days and then dried. Vertical bars represent mean ± SE. Levels were established both immediately (0 h) and 24 h after drying. The letters indicate significant differences (no drying and 0h: $F(1,8)=111.15,\ p<0.05$; 0 h and 24 h: $F(1,8)=93.11,\ p<0.05$; and no drying and 24 h: $F(1,8)=0.60,\ p=0.46$).

5.3.1.2.3 Phenolic acid and superoxide

Phenolic acid levels, from *in vitro E. grandis* shoots cultured on acclimation medium for 3 and 6 days, increased immediately after drying (0 h) (Fig 46). When the dried shoots were allowed to recover on acclimation medium for 12 h and 24 h, the phenolic acid levels decreased to similar levels, but these were less than the levels from the non-dried shoots (Fig 46). Culture duration had no impact on the phenolic acid levels (Fig 46).

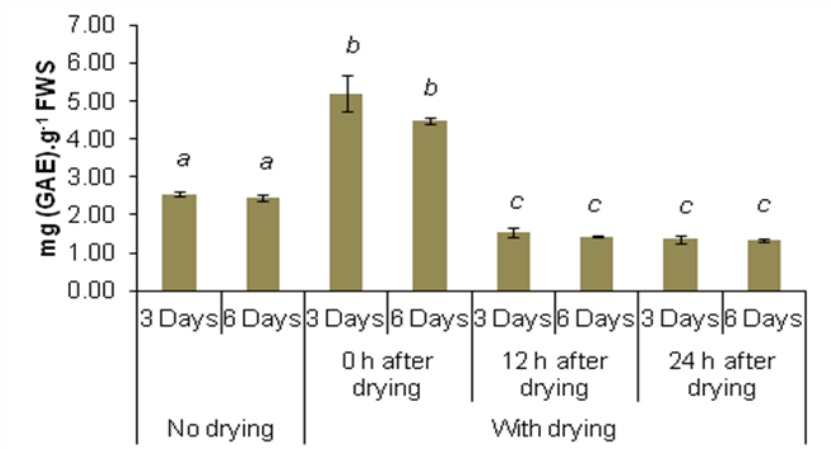


Fig 46. Phenolic acid (GAE) levels of *in vitro E. grandis* shoots cultured on acclimation medium for 3 or 6 days and then dried (with SE). Vertical bars represent mean ± SE. Levels were established immediately (0 h), 12 h and 24 h after drying. The letters indicate significant differences, according to post-hoc Tukey HSD at a 0.05 level of significance (no drying and 0 h: *F(1,8)=2.16, p=0.18*; 0 h, 12 h and 24 h: *F(2,12)=2.30, p=0.14*; and no drying and 24 h: *F(1,8)=0.34, p=0.58*).

Immediately after drying (0h) the superoxide levels were high in shoots that had been cultured for 6 days (0.181 ± 0.008 mg adrenochrome.g^{-1} FWS), whereas the level from shoots that had been cultured for 3 days remained statistically similar to levels from non-dried shoots (Fig 47). Even after 12 h and 24 h of recovery on acclimation medium, superoxide levels from dried shoots that had been cultured for 3 days remained similar to those of the non-dried shoots (Fig 47). In contrast, recovery of dried shoots that had been cultured for 6 days showed significant decreases in superoxide levels (Fig 47). However, the decreased level after 12 h of recovery was still high, compared with non-dried shoots and the recovered shoots that had been cultured for 3 days (Fig 47). Only after 24 h of recovery had the levels returned to pre-drying levels, albeit higher than the 3 day material (Fig 47).

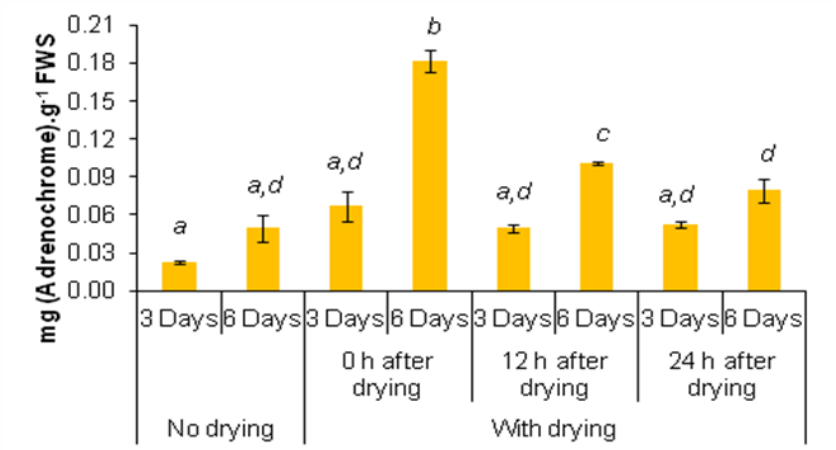


Fig 47. Superoxide (adrenochrome) levels of *in vitro E. grandis* shoots cultured on acclimation medium for 3 or 6 days and then dried. Vertical bars represent mean ± SE. Levels were established immediately (0 h), 12 h and 24 h after drying. The letters indicate significant differences, according to post-hoc Tukey HSD at a 0.05 level of significance (no drying and 0 h: $F(1,8)=24.23,\ p<0.05$; 0 h, 12 h and 24 h: $F(2,12)=20.26,\ p<0.05$; and no drying and 24 h: $F(1,8)=0.001,\ p=0.98$).

5.3.1.2.4 Proportions of biomolecules relative to the fresh weight of shoots (FWS)

Immediately after drying (0 h), the proportions of each biomolecule in shoots were different between the culture periods, whereas non-dried shoots appeared similar (Table 18). The starch levels immediately after drying decreased below 1% for both culture periods, while TSS proportions were higher than that in the non-dried shoots. Shoots that had been cultured for 3 days and then dried showed a TSS proportion approximately 2 times greater than shoots that had been cultured for 6 days and then dried (Table 18). The unknown component, immediately after drying, for the 6-day cultured shoots that were dried was nearly 8 times greater than the 3-day cultured shoots that were dried (Table 18). Although proline and superoxide were still below 1% immediately after drying, the proportions for shoots that had been cultured for 6 days were approximately 2 and 3 times (respectively) the proportions assayed in shoots that had been cultured for 3 days and then dried (Table 18). Phenolic acid were similar in proportions immediately after drying but greater than the non-dried shoots (Table 18). After 24 h of recovery, phenolic acid proportions had decreased below 1%, whereas proportions for other biomolecules increased to levels below what was observed with non-dried shoots, with exception to starch and unknown components which were higher than or similar to the proportions of non-dried shoots, all irrespective of culture periods (Table 18).

Table 18. Overall proportion of each class of biomolecule within the dry mass of *in vitro E. grandis* shoots cultured for 3 or 6 days on acclimation medium without drying and at 0 h and 24 h after 80 min of cryopreparative drying (with SE). The table represents the proportion (%) of biomolecules (mg biomolecules.g^{-1} fresh weight of shoots, FWS) relative to the mean dry mass of shoots (mg dry mass of shoots.g^{-1} FWS) for each treatment.

Proportion (%)	Without Cryopreparative Drying		With Cryopreparative Drying			
			After 0 h		After 24 h	
	3 Days	6 Days	3 Days	6 Days	3 Days	6 Days
TSS	15.38 ± 1.05	17.59 ± 2.33	89.61 ± 3.60	47.65 ± 1.90	18.67 ± 0.91	12.98 ± 0.77
Starch	3.27 ± 0.41	3.85 ± 0.05	0.891 ± 0.021	0.551 ± 0.123	4.73 ± 0.49	3.86 ± 0.12
Phenolic Acid	1.46 ± 0.04	1.64 ± 0.05	3.14 ± 0.24	3.09 ± 0.05	0.670 ± 0.042	0.618 ± 0.015
Superoxide	0.013 ± 0.001	0.033 ± 0.007	0.040 ± 0.007	0.125 ± 0.006	0.026 ± 0.001	0.031 ± 0.004
Proline	0.059 ± 0.005	0.120 ± 0.003	0.215 ± 0.012	0.490 ± 0.011	0.043 ± 0.002	0.069 ± 0.008
Unknown	79.81 ± 1.26	76.76 ± 2.34	6.11 ± 3.51	48.10 ± 1.86	75.85 ± 1.18	82.44 ± 0.67

5.3.2 DISCUSSION

Drying for 80 min and subsequent recovery on tissue culture medium affected both the physiological and biochemical responses of *in vitro E. grandis* shoots. Although ultimately the physiological and biochemical responses after drying and recovery were the same, the variation within the responses in some instances differed according to duration of the culture period (3 or 6 days) prior to drying.

5.3.2.1 Underlying physiological responses from shoots cultured for 3 or 6 days on acclimation medium and then dried

The culturing of shoots for 3 or 6 days on acclimation medium did not alter the majority of the physiological responses to 80 min of drying (Figs 39-41 and Table 16). The WC was reduced to levels that were statistically similar (Fig 39), and had achieved the low water content level for cryopreservation purposes (Padayachee *et al.*, 2008). Similarly for both culture periods, leaves of shoots immediately after drying demonstrated adaptive leaf movements (Figs 42B and E). The adaptive movements of folding or rolling of leaves are reversible mechanisms of dehydration avoidance, which help attenuate the impact of dehydration and excess radiation (Kadioglu & Terzi, 2007). The effect of leaf-rolling or -folding can reduce the leaf surface area exposed to radiation, and importantly reduce the transpiration rate by creating a microclimate that establishes high leaf humidity and a resistant vapour boundary layer on leaf surfaces, which prevents further loss of water (Hsiao *et al.*, 1984; Kadioglu & Terzi, 2007; Saglam *et al.*, 2008). The onset of rolling and folding are believed to be brought about by osmotic adjustments in leaves that are regulated by integrated molecular and plant growth regulator networks, which are initially triggered by decreases in relative water content coupled with high irradiation or temperatures (Kadioglu & Terzi, 2007). However, this physiological mechanism has been documented mostly in grass and cereal species e.g. *O. sativa* (Hsaio *et al.*, 1984; Kadioglu & Terzi, 2007), and the rhizome-forming herbaceous plant *Ctenanthe setosa* (Saglam *et al.*, 2008), but not in woody species.

Despite the adaptive leaf movements and the recovery of WC levels after drying (Fig 39), the viability of shoots after 2 weeks of recovery were reduced (Table 16). As the shoots succumbed to the severe dehydration stress, most of the original leaves senesced and browned (Figs 42C and F). The ability to recover WC was similarly observed with pubesecent oak trees (Galle *et al.*, 2007) and *E. pauciflora* (Kirschbaum, 1988) seedlings,

where leaf water status rapidly recovered upon re-watering. Although there was widespread senescence and browning, it would appear that the survival of axillary buds was ensured through underlying abiotic stress-related biochemical responses (Figs 42C and F). Leaf senescence is a sequential process of molecular, biochemical and physiological events that is seen as the final stage of development in cells and tissues that are preparing for death, when mature regions of the plant are beyond their assimilatory capacity as a consequence of age or imposition of stress (Smart, 1994). In brief, senescence involves the dismantling of photosynthetic systems, and at the same time active metabolic regulation, where nutrients are exported to young growing tissues or storage organs (e.g. dormant buds) (Smart, 1994). This ensures the survival of other plant organs and minimizes ROS overproduction (Smart, 1994). Leaf senescence has been recorded in other *Eucalyptus* and woody species responding to severe dehydration stress (Gindaba *et al.*, 2004; Merchant *et al.*, 2007; and see Section 5.1).

Senescence may have therefore ensured survival of specific tissue/-organs in the *in vitro E. grandis* shoots during recovery; these were predominantly axillary buds that slowly grew and developed leaves and shoots (Figs 42C and F). Although the responses after drying and recovery were common for both culture periods (Figs 42C), the development of axillary buds differed, where 6-day cultured shoots appeared more developed (Figs 42C and F). This suggested that culturing for 6 days may have helped with preserving the integrity of these axillary buds, which may be attributed to the more adaptable primary metabolic status of shoots prior to drying. In this regard, the axillary buds could have employed a more diversified abiotic stress-related biochemical response. Thus, the culturing period may indicate the effects of age on responses to dehydration stress (Chaves *et al.*, 2009; Merchant *et al.*, 2007; and see Section 5.1). However, shoots during recovery still showed very slow signs of growth and development (Figs 42C and F), which again substantiated the severity, long-lasting and damaging effects of this drying regime on shoots.

Apart from the slow signs in growth and development, newly developing leaves and tissues from axillary buds were mainly shades of light green (Figs 42C and F), and in some cases there were signs of visible red pigments on the underside of some leaves (Fig 42F), similar to the observations immediately after drying (Fig 42E). The light green colouration implied that chlorophyll content was low, and suggested that the already reduced photosynthetic capacity remained severely affected and/or still in the process of being partially re-established. The light green colour could also indicate sustained oxidative stress and resultant photoprotection that continued even after drying (Chaves *et al.*, 2009). In this respect, *E. pauciflora* seedlings

for instance showed lowered carbon dioxide assimilation rates when exposed to severe dehydration stress, which was slightly depressed by photoinhibition (Kirschbaum, 1987). This was also similarly observed with pubescent oak trees (Galle *et al.*, 2007). The reddish pigmentations were a sign of juvenile reddening or a loss of chlorophyll during senescence, and involved the accumulation or revealing of anthocyanins and carotenoids for photoprotection (Chalker-Scott, 1999; Steyn *et al.*, 2002). Accumulation of anthocyanins and carotenoids are essential for juvenile plants i.e. young leaves (Fig 42F) and even senescing leaves (Fig 42E) because these types of tissues have lowered abilities to utilize absorbed light energy (Chalker-Scott, 1999; Steyn *et al.*, 2002). Anthocyanins and carotenoids are also accumulated when dehydration stress is experienced (Chalker-Scott, 1999; Merchant *et al.*, 2007; Steyn *et al.*, 2002). This therefore makes photosynthesis in these tissue types more susceptible to photoinhibition and photobleaching than mature and pre-senescing leaves, and as a consequence would lead to secondary oxidative stress (Chalker-Scott, 1999; Steyn *et al.*, 2002). As such, this sharply reduces photosynthetic capacity and causes the inherent degradation of photosynthetic pigments because of the over-absorbance of light energy (Chalker-Scott, 1999; Merchant *et al.*, 2007; Steyn *et al.*, 2002; and see Section 4.3.1).

5.3.2.2 Underlying biochemical responses from shoots cultured for 3 or 6 days on acclimation medium and then dried

Although viabilities were reduced by drying, the majority of the *in vitro E. grandis* shoots survived. This survival of shoots can be attributed to the complex mechanisms of tolerance that were triggered by the dehydration stress. These tolerance mechanisms are brought about by underlying abiotic stress-related biochemical responses, induced by molecular and signalling responses (abiotic stress-related molecular and signalling pathways triggered by sensors) to drying (Fujita *et al.*, 2009; Gaspar *et al.*, 2002; Kaplan *et al.*, 2004; Seki *et al.*, 2007; Shinozaki *et al.*, 2003). Despite the commonality of biochemical responses employed to tolerate drying, the contribution of these responses in some instances differed according to the duration of the culturing periods, which could also explain the subtle differences in physiological responses.

5.3.2.2.1 Starch and soluble carbohydrate responses

Irrespective of the culture duration (3 or 6 days), drying the *in vitro E. grandis* shoots may have commonly triggered metabolic activities involved in the conversion of starch into soluble carbohydrates and/or also promoted synthesis of soluble carbohydrates (Figs 43 and

44). This trend was similarly reflected in other studies that imposed gradual soil water deficits on whole plant systems (e.g. seedlings and rooted cuttings). Different *Eucalyptus* species were shown to have accumulated soluble carbohydrates (e.g. sucrose, fructose and glucose) when experiencing dehydration stress, and this was attributed to increased soluble carbohydrate metabolic activities accompanied by the possible breakdown of starch (Shvaleva *et al.*, 2006; Warren *et al.*, 2011a,b). Other plant species such as *Populus*×*euramericana* clones, *Coffea canephora* and the model-plant *A. thaliana* also reflected similar responses towards dehydration stress (Hummel *et al.*, 2010; Marron *et al.*, 2002**;** Praxedes *et al.*, 2006). However, the TSS levels indicated that *E. grandis* shoots pre-cultured for 3 days may have depended predominantly on soluble carbohydrates for tolerance, whereas the shoots pre-cultured for 6 days relied more on a diverse combination of biochemical responses (Fig 44). The effect of plant age on the levels of dehydration tolerance and survival was also demonstrated between 4-day-old autotrophic and 6-day-old heterotrophic wheat seedlings, where survival was related to the distribution and composition of soluble sugar pools (Bogdan & Zadanska, 2006).

When the *E. grandis* shoots were recovered for 24 h on acclimation medium, the recovery process may have commonly triggered metabolic activities involved in the re-conversion of soluble carbohydrate into starch, irrespective of the pre-culture period (Figs 43 and 44). This reversible carbon partitioning was also suggested during re-watering of the seedlings of different *Eucalyptus* species, where fructose and glucose levels decreased, while sucrose remained similar to the level under water deficit (Warren *et al.*, 2011a,b). A decrease in soluble sugars was also observed with rooted-cuttings of *Populus*×*euramericana* clones when re-watered after imposed drought conditions, and returning to similar levels as the controls (Marron *et al.*, 2002). Although recovery responses after drying were common in both culturing periods, TSS levels remained higher in shoots pre-cultured for 3 days (Fig 44), further supporting the main dependency on starch and carbohydrate for tolerance. Higher starch levels in shoots after recovery suggested that there were soluble carbohydrates synthesised during drying (Figs 43 and 44), further supporting the notion that there was drying stress-induced carbohydrate synthesis. In addition, it may be possible that the transient conversion into starch during recovery could have partially contributed into the controlled uptake of water, ensuring that WC returned to optimal levels and that accessible starch reserves were available for metabolic recovery (Fig 39). However, this would need to be experimentally verified as there is a lack of indicative information in that regard.

This strong dependency of 3-day cultured shoots on carbohydrates could be attributed to the metabolic state of the shoots prior to drying. The metabolism of 3-day cultured shoots might still have been under the influence of residual metabolites of exogenous PGRs that were in the process of becoming slowly depleted (see Sections 3.1 and 3.3). This response therefore suggested that the metabolic state of *in vitro E. grandis* shoots was still changing from multiplication-orientated growth prior to drying. Obviously, this also would need to be experimentally verified. Shoots that had been cultured for 6 days, on the other hand, may have been in a non-PGR induced metabolic state with reduced multiplication-related carbohydrate metabolism and involvement of other metabolic activities. As a result, 6-day cultured shoots may have had a more adaptable metabolism to drying, in contrast to the carbohydrate-dependent response of 3-day cultured shoots.

5.3.2.2.2 Proline responses

Culturing of shoots for 6 days promoted the accumulation of proline during a non-PGR induced metabolic state (see Section 3.3), but only after drying did the stress-related role of proline become more evident at both culture periods, since the levels in shoots increased (Fig 45). Accumulation of proline was also observed in seedlings of other *Eucalyptus* species that experienced dehydration (Shvaleva *et al.*, 2006; Warren *et al.*, 2011b). The rapid accumulation of proline is also a common response shared with many other agricultural plant species that were exposed to dehydration, such as in sunflower (Manivannan *et al.*, 2007), eggplant (Sarker *et al.*, 2005), tobacco (Dobra *et al.*, 2011), pea (Sanchez *et al.*, 1998), and young carambola plants (Ismail *et al.*, 1994), including the model-plant *A. thaliana* (Hummel *et al.*, 2010; Rizhsky *et al.* 2004). Warren *et al.* (2011a) showed that other amino acids were also accumulated (tryptophan, tyrosine, serine, glycine, threonine, and phenylalanine) in other *Eucalyptus* species experiencing dehydration stress. The *E. grandis* shoots pre-cultured for 6 days had the highest level of proline content (Fig 45), which implied that drying tolerance in these shoots strongly required this amino acid. Even after 24 h of recovery, when proline levels had decreased in shoots from both culture periods, the level from 6-day cultured shoots remained high (Fig 45). Rapid reduction of proline levels during recovery is important because an excess of proline and its conversion product pyrroline-5-carboxylate are toxic (Lehmann *et al.*, 2010; Trovato *et al.*, 2008). It has been suggested that these molecules can participate in the production of ROS and in inducing apoptosis and programmed cell death (Lehmann *et al.*, 2010; Trovato *et al.*, 2008). The higher proline level after recovery from shoots that had been cultured for 6 days further substantiated the importance of the

amino acid in osmoprotection even during recovery, and also indirectly suggested the susceptibility of these shoots to drying (Fig 45).

5.3.2.2.3 Phenolic acid and superoxide responses

In response to drying, phenolic acid synthesis was promoted in shoots irrespective of culture period (Fig 46). High phenolic acid content and even increased pre-cursor biomolecules of phenolic acid synthesis were also observed in other plant and *Eucalyptus* species. Some *Eucalyptus* species that experienced dehydration showed increases in amino acids (phenylalanine and/or tryptophan and/or tyrosine) involved in phenylpropanoid biosynthesis and decreases in catechin, a precursor of lignin synthesis (Warren *et al.*, 2011a). This indirectly indicated the possibility of promoted metabolism, or even reduced activity of enzymes (Warren *et al.*, 2011a). Foliage of drought-adapted *E. nitens* (Close & McArthur, 2002), mature foliage of *Populus*×*euramericana* (Dorskamp clone) (Marron *et al.*, 2002) and other plants also showed high accumulation of phenolic acid and the end-product anthocyanins after experiencing dehydration stress (Chalker-Scott, 1999; Steyn *et al.*, 2002). However, whether dehydration by itself is the cause for increased phenolic acid accumulation has been questioned, since environmental stresses like the dehydration stress of drought ultimately impacts photosynthesis by reducing its photosynthetic capacity (Grace, 2005; Jones & Hartley, 1999). A reduced photosynthetic capacity combined with light would increase the potential of photoinhibition, and because of this phenylpropanoid accumulation would be promoted (Grace, 2005; Steyn *et al.*, 2002).

Nonetheless, the high phenolic acid level and other stress-related biomolecules in the *E. grandis* shoots may have minimized superoxide to metabolically tolerable levels in the 3-day cultured shoots that were dried, whereas 6-day cultured shoots showed elevated levels of both phenolic acid and superoxide (Figs 46 and 47). Metabolism of the 6-day cultured shoots may have been more susceptible to the damages and injuries caused by drying, and this resulted in an overproduction of superoxide from decoupled pathways (Fig 47), even though other stress-related biomolecules were present. However, a comparable measure of metabolism (e.g. photosynthetic rate and enzymatic activities) would need to be performed between the two culture periods for the shoots in order to confirm this susceptibility. Furthermore, the synthesis of phenolic acid is dependent on enzymatic activities (Dixon & Paiva, 1995; Grace, 2005; Solecka, 1997), thus these enzymes would be limited by their rate

constants, whereas the overproduction of superoxide is a continuous process from decoupled metabolic systems (Gill & Tuteja, 2010).

After 12 h of recovery on medium, the 6-day cultured shoots showed decreased superoxide levels, but only after 24 h of recovery were the metabolically tolerable levels re-achieved (Fig 47). In contrast, the 3-day cultured shoots demonstrated no response to the drying and maintained tolerable superoxide levels even after 12 h and 24 h of recovery. However, the low superoxide levels may have also indicated that many metabolic processes were already not functioning, since a majority of cells and tissues may have already died in shoots, but this would have to be experimentally verified. Minimizing superoxide levels may well have involved the action of the antioxidant phenolic acid, but synthesis of phenolic acid might have been suppressed during recovery and repair, since levels were far less than levels from non-dried shoots, irrespective of culturing periods (Figs 46 and 47). Similarly, this trend was observed with the Dorskamp clone of *Populus*×*euramericana*, where re-watering after dehydration resulted in decreases in total phenol in mature leaves, and these phenolic compounds were suggested to be partially responsible for antioxidant activity (Marron *et al.*, 2002). It was also suggested by Warren *et al.* (2011) that re-watering would have reversed the changes of phenylpropanoid metabolism in the *Eucalyptus* species. This implied that phenolic acid synthesis was suppressed to a minimum because metabolism was re-directed towards repair and recovery. According to the protein competition model for phenolic allocation, it was suggested that total phenolic allocation competes with protein synthesis, since both require amino acids (e.g. phenylalaine) as pre-cursors (Jones & Hartley, 1999). The model predicted an inverse relationship between total phenolic allocation and total protein allocation, according to different developmental stages of plants and the environmental conditions encountered (Jones & Hartley, 1999). It is therefore possible that amino acids were directed predominantly to protein synthesis, while phenylpropanoid metabolism input was reduced during recovery of the *E. grandis* shoots. Determination of protein content after drying and recovery would verify this. It is also possible that the low phenolic acid levels after recovery was due to a leaching effect caused by the acclimation medium, where phenolic acid had slowly leached into the surrounding medium, which is a common observation in our laboratory (Fig 48) and a well-know occurrence for *in vitro* culture (Peerce, 2008). The phenolic acid may have been leached from membranes of plant cells that could have become leaky as a consequence of the damaging effects of drying, thereby blackening the medium (Peerce, 2008).

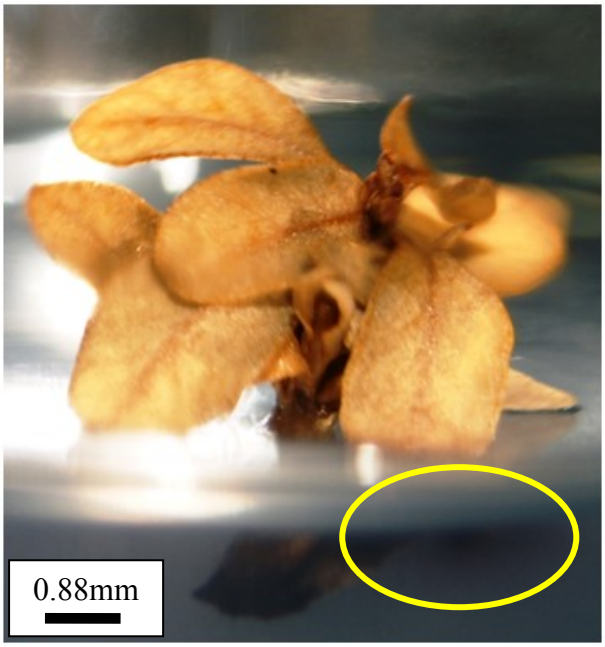


Fig 48. A non-viable *in vitro E. grandis* shoot showing the leaching of phenolic acid into the multiplication medium, during recovery. Yellow circle indicate leached phenolic acid, which is identified as a black shadow/darkening in the medium surrounding the shoot.

5.3.2.2.4 Indications from the contribution of biomolecules to the fresh weight of shoots (FWS)

Tolerance of *in vitro E. grandis* shoots strongly depended on starch and soluble carbohydrate metabolism (Table 18). This dependency was also similarly identified in other *Eucalyptus* (Shvaleva *et al.*, 2006; Warren *et al.*, 2011a,b) and other plant species (Marron *et al.*, 2002; Sanchez *et al.*, 1998). As such tolerance to dehydration stress can be seen to be dependent on primary carbon metabolism and partitioning for osmotic protection (Warren *et al.*, 2011a,b; Section 5.1). In addition, Warren *et al.* (2011a,b) also indicated that shikimic acid was closely associated with adaptive primary carbon metabolism; this is an intermediate pre-cursor in the phenylpropanoid pathway for phenolic compounds.

Another observation in the current investigation was that the culturing period prior to drying influenced the extent of dependency of shoots on carbohydrates (Table 18). The 3-day cultured shoots strongly depended on soluble carbohydrates, followed by phenolic acid and proline (Table 18 and Figs 44-46). With regard to proline, it has been suggested that this

plays a secondary role in osmoprotection and not in osmotic adjustments (Sanchez *et al.*, 1998; Shvaleva *et al.*, 2006), since its contribution to the osmotic potential of leaves was less than 1% in water-stressed *E. globulus* clones (Shvaleva *et al.*, 2006). In contrast, 6-day cultured shoots depended on a more diversified combination of carbohydrates, phenolic acid, proline and other unknown components (Table 18 and Figs 44-46) and therefore tolerance was achieved through the activities of several abiotic stress-related biochemical responses.

When shoots were recovered for 24 h on acclimation medium, biomolecule proportions had returned to metabolic states that resembled non-dried shoots (Table 18). This reversal of biochemical changes was similarly observed in other *Eucalyptus* species when re-watered (Warren *et al.*, 2011a,b). These proportional changes further substantiated the role of these biochemical responses in tolerance (Section 5.3.2.2.1-3). The recovered 3-day cultured shoots had high starch proportions, which supported the notion that there may have been more stress-related soluble carbohydrates synthesised during drying (Table 18). The recovered shoots also suggested that there were re-allocations of soluble carbohydrates and biomolecules to the unknown component portion, which may indicate that metabolism returned to similar states prior to drying (Table 18). On the other hand, the recovery of 6-day cultured shoots resulted in a higher proportion of unknown components (Table 18), suggesting that there may be additional undetermined biochemical responses involved in recovery and repair. In contrast, the recovered 3-day cultured shoots had a lower unknown component portion (Table 18), which may indicate that biochemical responses were reduced by the effects of dehydration stress. Furthermore, the above mentioned trend was also reflected in contrasting *Eucalyptus* species, where re-watering after experiencing dehydration stress resulted in additional increases of measured total metabolites in *E. dumosa* seedlings, whereas *E. pauciflora* showed decreased total measured metabolites (Warren *et al.*, 2011b).

5.4 SUMMARY AND CONCLUSION

The response of the *in vitro E. grandis* shoots clearly demonstrated that tolerance towards physical drying required a combination of biochemical responses. This indicates that even a sub-tropical woody species such as *E. grandis* depends on the activities of these biomolecules (particularly starch and carbohydrates) for surviving dehydration stress. This further substantiated that irrespective of plant type and species, these biomolecules are important during tolerance towards dehydration stress, where tolerance (e.g. osmotic adjustments) depends strongly on soluble carbohydrates.

Specific to this part of the study, the contributions of these biomolecules to tolerance towards dehydration stress depended on the diversity of biochemical responses from *in vitro E. grandis* shoots (see Section 5.3.2.2.4). This is further substantiated by literature, which strongly support that adaptive tolerance of plants to dehydration stress is not conferred by a specific resistance mechanism (biochemical or physiological), but a simultaneous interplay of several mechanisms, where contributions of each mechanism to tolerance differ according to species and stress conditions (Chaves *et al.*, 2003; Shvaleva *et al.*, 2006). This diversity of biochemical responses may well have been influenced by the presence of residual synthetic PGRs during the 3 and 6 day culturing periods on acclimation medium. *In vitro E. grandis* shoots cultured for 3 days may have had remaining residual PGR-related metabolites, which limited metabolism to growth and development (see Sections 3.3 and 5.3.2.2.1). Thus, tolerance was strongly dependent on carbohydrate metabolism (associated with growth), while phenolic acid and proline were involved to a lesser extent (see Section 5.3.2.2.1). In contrast, 6-day cultured shoots may have depleted residual PGR-related metabolites (see Section 5.3.2.2.2), which allowed a more diverse range of biochemical responses for tolerance. This included other unknown biochemical components, but tolerance still remained strongly dependent on carbohydrate metabolism (see Section 5.3.2.2.4). However, irrespective of the culture periods, none of the biochemical responses could prevent nor protect against all of the damage incurred during drying, although some shoots still remained viable (Table 16, and see Sections 5.3.1.1 and 5.3.2.1).

The *in vitro E. grandis* shoots that survived showed severely impacted vigour, with both the 3-day and 6-day cultured shoots displaying similar vigour responses (Figs 40 and 41, and Table 17). However, the morphological development of shoots during recovery from 6-day cultured shoots was better than the 3-day cultured shoots (Fig 42; and see Section 5.3.1.1), despite the greater oxidative stress that may have been experienced by the 6-day cultured shoots (Table 18 and Fig 47; and see Section 5.3.2.2.3). This could be attributed to a more diverse combination of biochemical responses conferring tolerance in the 6-day cultured shoots (see Section 5.3.2.2.4). Nonetheless, shoots dried for 80 min achieved the water contents required for cryopreservation (Figs 35 and 39). It was therefore important to consider whether it was possible to achieve such preferable water content without compromising their viability.

CHAPTER 6.

Cross-tolerance in *Eucalyptus*? Some physiological and biochemical responses of *in vitro E. grandis* shoots to cold acclimation and physical drying

6.1 INTRODUCTION AND LITERATURE REVIEW

Upstream molecular investigations of 'cross-talk' in plants at a genomic, transcriptional and hormonal level have converged in suggesting the existence of the downstream response of 'cross-tolerance'. Some examples of 'cross-tolerance' expressed in plants (often seedlings) exposed to different combinations of abiotic and biotic stresses are presented in Table 19. In addition, genomic investigations on the model-plant *Arabidopsis thaliana* have also suggested this phenomenon, where specific genes involved in adaptive mechanisms of tolerance have been shown to be commonly induced by various individual abiotic stresses (Rizhsky *et al.*, 2004; Seki *et al.*, 2002). For example, *A. thaliana* seedlings exposed to drought, cold and high-salinity stresses upregulate forty-three genes involved in a variety of biochemical responses imparting tolerance (Seki *et al.*, 2002). These examples therefore provide substantial evidence based on specific physiological and biochemical responses, highlighting the phenomenon 'cross-tolerance'.

Table 19. Examples of 'cross-tolerance' response to abiotic and/or biotic stresses

Species	Plant system	Growth condition	Pre-treatment or acclimation condition	'Cross-tolerance' stress	Reference
Triticum aestivum L. (cv. Cheyenne and Chinese spring)	Seedlings	Hydroponic	Cold acclimation: 2 days at 5°C Oxidative acclimation: 0.1mM hydrogen peroxide for 1 day	Drought stress: induced by 1 day exposure to 15% polyethylene glycol Drought stress or freezing stress: -11°C for 1 day	Horvath *et al.*, 2007
Polish Festulolium (cv. Rakopan): genotypes 561 and 621	Field cuttings	Greenhouse	Pre-hardening for 2 weeks at 12°C and hardening for 3 weeks at 2°C	Artificial inoculation with *Microdochium nivale* (pink snow mould)	Pociecha *et al.*, 2009
Glycine max L. Merrill (cv. Maple Arrow and Fiskeby V)	Seedlings	Growth chamber	Drought stress: 3 days of no irrigation (28°C day/23°C night)	Drought stress, and dark chilling (28°C day/8°C night) for 9 days	Riekert Van Heerden & Kruger, 2002

Table 19. Continued

Chenopodium quinoa Willd.	Seedlings	Growth chamber	Salt-acclimation: 200mM NaCl saline treatment	Salt-acclimation continued to low temperature stress: 5°C/5°C (day/night)	Rosa *et al.*, 2009
T. aestivum L. (cv. Zimai 1)	Seeds	Pedri dish germination in 5 days	Salt-acclimation: -0.8MPa NaCl solution at 20°C Heat stress: 33°C	Heat stress: 33°C Salt-acclimation: -0.8MPa NaCl solution at 20°C	Song *et al.*, 2005

In the model-plant *A. thaliana*, the prominent role of carbohydrate metabolism in tolerance to simultaneous exposure to abiotic stresses has also been emphasised both genetically and metabolically. There were several functional genes related to carbohydrate metabolism and osmoprotectant synthesis observed to be commonly upregulated by drought, cold and high-salinity stresses in *A. thaliana* seedlings (Seki *et al.*, 2002). This genomic upregulation was evidenced downstream by the higher accumulation of several different soluble carbohydrates when seedlings were simultaneously exposed to both drought and heat, compared with dehydration stress alone (Rizhsky *et al.*, 2004). In addition, genomic evidence also showed that several genes encoding phenylpropanoid enzymes (e.g anthocyanidin synthase, cinnamyl-alcohol dehydrogenases, and cinnamoyl-CoA reductase) were commonly up-regulated under low temperature and dehydration stress (Seki *et al.*, 2002).

The prominent role of carbohydrate metabolism was also demonstrated in cotyledons of quinoa that were salt-acclimated and then transferred to low temperature stress or were simultaneously salt- and low temperature-stressed. These abiotic stress treatments resulted in sucrose-starch partitioning that was characterised by a rapid decrease in starch levels, while sucrose, glucose and fructose levels increased (Rosa *et al.*, 2009). Changes in source-sink relation were inferred to have supplied soluble carbohydrates for osmotic adjustments, despite the similarity of this accumulation with the levels in the cotyledons that were only exposed to low temperature stress (Rosa *et al.*, 2009). In addition to soluble carbohydrates, the accumulation of proline for the first six days in cotyledons of quinoa that were salt-acclimated and then transferred to low temperature stress were higher than those directly transferred to simultaneous salt- and low temperature-stress conditions (Rosa *et al.*, 2009). However, after the six days, proline accumulation under these abiotic stress conditions reversed where the latter became far greater than the former (Rosa *et al.*, 2009). In soybean

seedlings, accumulation of proline was induced by drought stress when pre-dawn leaf water potential was at -0.6 and -0.8MPa. In contrast, the simultaneous abiotic stress conditions (chilling in the dark and drought) had delayed proline accumulation in seedlings until pre-dawn (i.e. dark) leaf water potential was less than -1.0MPa (Riekert Van Heerden & Kruger, 2002). These different responses suggested that the dependency on proline could be related to a reciprocal relationship between the amino acid accumulation and the presence of other osmolytes that compensated for the initial lack of proline synthesis for tolerance (Riekert Van Heerden & Kruger, 2002).

'Cross-tolerance' has been reported for in a number of other species. For example, in Polish Festulolium cuttings (cultivar Rakopan), cold acclimation improved the resistance of the genotype to both frost and infection by the snow mould, *Microdochium nivale* (Pociecha *et al.*, 2009). This improved resistance was attributed to cold acclimation inducing a higher phenolic content before infection, which interestingly coincided with high ABA concentration and low water potential in the cuttings (Pociecha *et al.*, 2009). The pre-treatment of *Triticum aestivum* (wheat) seedlings (cv. Cheyenne) with drought stress for 1-day increased the subsequent tolerance and survival to freezing (Horvath *et al.*, 2007). Similarly, wheat seedlings that had undergone two days of cold acclimation showed improved tolerance and survival to severe dehydration stress, which was in part attributed to increased antioxidant enzyme activities (guaiacol and ascorbate peroxidase) promoted during cold acclimation (Horvath *et al.*, 2007). Although not a mature plant, the germination of wheat under 116h of salinity stress (-0.8MPa NaCl) improved when seeds were pre-treated to heat stress (33°C) for 4h (Song *et al.*, 2005).

Although it is clear that plants are capable of 'cross-tolerance', a survey of the literature has yielded neither reports for 'cross-talk' nor 'cross-tolerance' in clonal *in vitro E. grandis*. The prospect of inducing 'cross-tolerance' in vegetative *in vitro Eucalyptus grandis* shoots to better survive the pre-treatment stage of physical drying during the cryopreservation process was therefore a possibility. Thus, it was the purpose of this study to investigate whether 'cross-tolerance' could be induced in *E. grandis* for cryopreservation purposes. In this regard, *in vitro E. grandis* shoot clusters were pre-treated to the appropriate chilling treatment of 10°C for 3 days and then physically dried for 80 min over activated silica gel (Chapter 4 and 5).

6.2 RESULTS

6.2.1 Physiological responses

Immediately (0 h) after drying, the WC of the shoots pre-treated at 10°C for 3 days was greater (0.52 ± 0.05 g water.g^{-1} FWS) than the control (25°C) dried material (0.32 ± 0.04 g water.g^{-1} FWS) (Fig 49). When dried shoots were recovered on acclimation medium for 24 h, the WC for both treatments returned to pre-drying levels. The WC between control and shoots pre-treated at 10°C for 3 days without drying and after recovery remained similar (Fig 49).

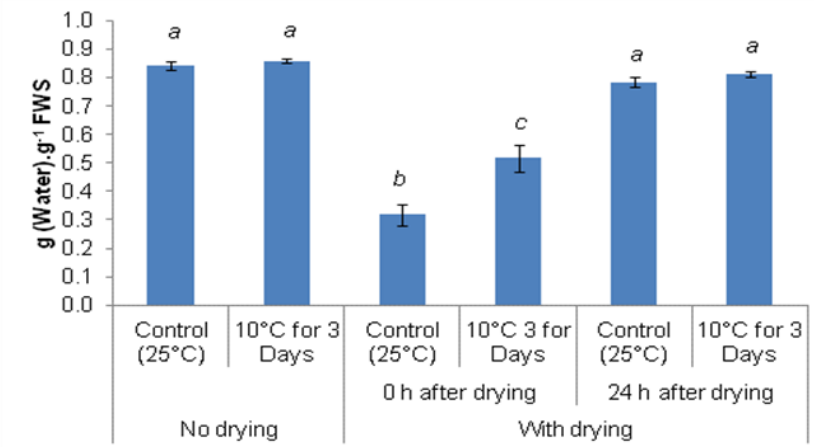


Fig 49. Water content (WC) of *in vitro E. grandis* shoots that were pre-treated to 10°C for 3 days and then dried for 80 min. Vertical bars represent mean ± SE. The letters indicate significant differences in WC levels (no drying and 0 h: $F(1,8)=8.53$, $p<0.05$; 0 h and 24 h: $F(1,8)=7.27$, $p<0.05$; and no drying and 24 h: $F(1,8)=0.13$, $p=0.72$.

The 10°C, 3 day treatment had no impact on viability (Table 20). Following 80 min of drying, the viability of control shoots decreased after 2 weeks on acclimation medium (88.9 ± 3.9%), whilst the shoots pre-treated to 10°C for 3 days remained fully viable (Table 20).

Table 20. Viability of *in vitro E. grandis* shoots that were pre-treated at 10°C for 3 days and then exposed to 80 min of cryopreparative drying. The viability of the control and shoots pre-treated to 10°C for 3 days without and with 80 min of cryopreparative drying are presented.

		Without Cryopreparative Drying		With Cryopreparative Drying	
	Week	Control (25°C)	10°C 3 Days	Control (25°C)	10°C 3 Days
Viability (%)	0	100	100	100	100
	2	100	100	88.9 ± 3.9[ab]	100

[a] Evaluated based on the number of viable shoots in a sample of 15: n=13.3 ± 0.3 for 6 Days

[b] Standard deviation

The trend of improved tolerance to drying as a result of the pre-treatment was also noted in the vigour data. While the vigour of dried shoots had significantly decreased compared with non-dried shoots (Figs 50 and 51), the growth of shoots in the experiment (shoots pre-treated at 10°C for 3 days and then dried) (2.8 ± 0.1 visible axillary buds/week) was significantly higher than the control (25°C) (1.9 ± 0.3 visible axillary buds/week) that was dried. The emergence of axillary buds, however, remained statistically similar between the control and experiment that were dried (Fig 50). In addition, callus formation in shoots was reduced by drying, whilst browning was promoted after 2 weeks of evaluation.

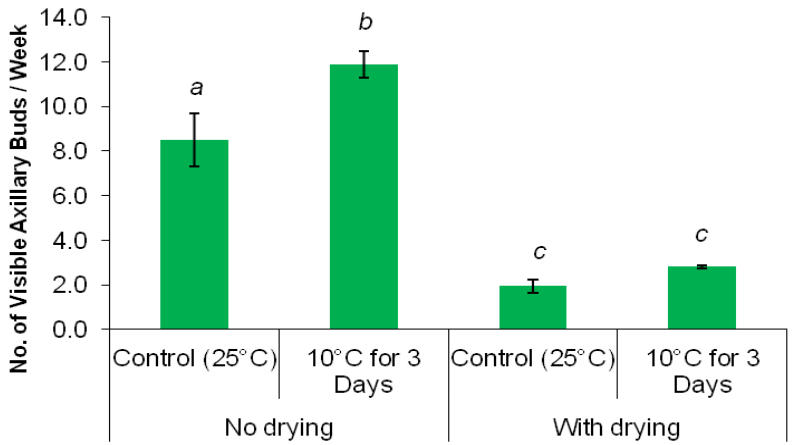


Fig 50. Vigour, in terms of the emergence of axillary buds, of *in vitro E. grandis* shoots pre-treated to 10°C for 3 days and then dried for 80 min. Vertical bars represent mean ± SE. The letters indicate significant differences, according to post-hoc Tukey HSD (0.05 level of significance, *F(1,8)=3.37, p=0.10*).

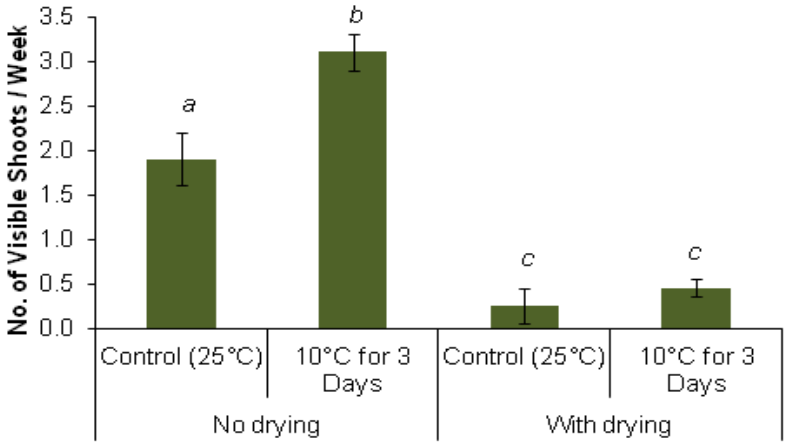


Fig 51. Vigour, in terms of the growth of shoots, of *in vitro E. grandis* shoots pre-treated to 10°C for 3 days and then dried for 80 min. Vertical bars represent mean ± SE. The letters indicate significant differences (*F(1,8)=6.12, p<0.05*).

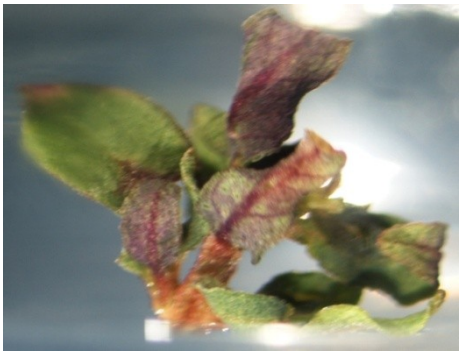
Table 21. Callus formation and tissue browning scores of *in vitro E. grandis* shoots that were pre-treated to the chilling and exposure treatment of 10°C for 3 days and then dried for 80 min (with SE). The scores of the control and 10°C, 3 day treatment for without and with 80 min of cryopreparative drying are presented. Callus formation was significantly higher in non-dried shoots than dried shoots (*F(1,31)=19.60, p<0.05*), while tissue browning was higher in dried shoots than non-dried shoots (*F(1,31)=30.67, p<0.05*).

Score (0-5)	Week	Without Cryopreparative Drying		With Cryopreparative Drying	
		Control (25°C)	10°C 3 for Days	Control (25°C)[a]	10°C 3 for Days
Callus Formation	0	0.2 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.1
	1	0.8 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.2
	2	1.2 ± 0.1	0.9 ± 0.0	0.4 ± 0.1	0.5 ± 0.2
Tissue Browning	0	0.8 ± 0.1	0.3 ± 0.2	0.8 ± 0.1	0.1 ± 0.1
	1	1.0 ± 0.0	0.8 ± 0.1	3.5 ± 0.2	2.9 ± 0.1
	2	1.1 ± 0.0	0.9 ± 0.1	3.3 ± 0.0	2.2 ± 0.1

[a] Evaluated based on the number of viable shoots in a sample of 15: n=13.3±0.3 for 6 Days

Also note worthy were the morphological contrasts observed from dried shoots after 2 weeks on multiplication medium (from the viability and vigour evaluations), when compared with non-dried shoots (Table 22). Immediately after drying, both the control and experimental material showed reddish-pigmentation on the stems and underside of the leaves, and this was accompanied with wrinkling and rolling of leaves (Table 22). After 2 weeks on multiplication medium, the growth of the control (no chilling treatment) and experiment that were dried were greatly reduced, compared with non-dried shoots (Table 22). The leaves and stems of the dried shoots after 2 weeks were pale green and this was accompanied by extensive browning of leaves, when compared with the darker green and minimal spread of browning of non-dried shoots (Table 22). The physical appearance of the shoots of the controls and 10°C, 3 day treatments, regardless of drying, were similar (Table 22). Non-dried shoots showed similarities in their growth, and dried shoots showed prominent axillary bud emergence as already noted in Fig 50 (Table 2).

Table 22. Representation of the physical appearance of *in vitro E. grandis* shoots (10× magnification) that were pre-treated to 10°C for 3 days and then exposed to 80 min of cryopreparative drying. Week 0 and 2 are presented for the dried shoots of the control (25°C) and 10°C, 3 day treatment, as well as week 2 for non-dried shoots. Week 0 shoots were photographed while shoots were within culture tubes, while week 2 shoots were photographed after removal from culture tubes. The scale for week 0 is 10:0.61mm, and week 2 is 10:0.88mm.

	Week	Control (25°C)	10°C for 3 Days
No Drying	2		
After Drying	0		
	2		

6.2.2 Biochemical responses

6.2.2.1 Starch and TSS

Drying significantly reduced starch levels within the shoots of both the control and experiment i.e. pre-treated material (Fig 52). Following 24h of recovery on acclimation medium, starch levels in the control shoots that were dried returned to levels statistically similar to the non-dried shoots (Fig 52), while the level within the experimental material was significantly higher (11.77 ± 0.83 mg starch.g^{-1} FWS) (Fig 52).

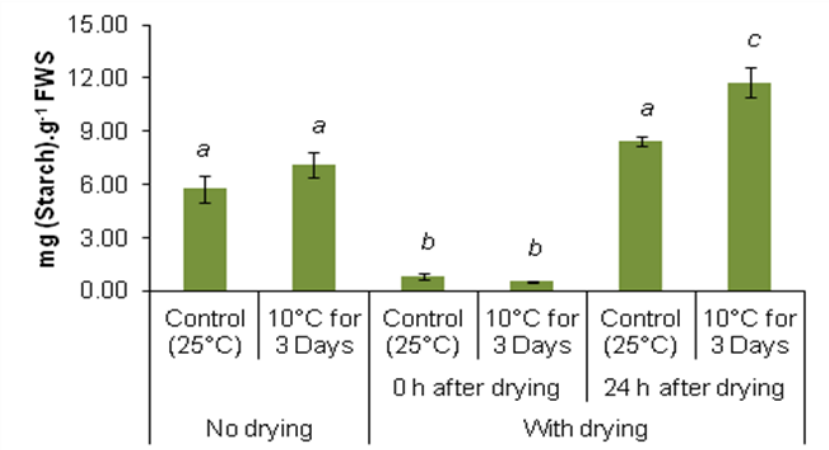


Fig 52. Starch levels of *in vitro E. grandis* shoots that were pre-treated at 10°C for 3 days and then dried for 80 min. Vertical bars represent mean ± SE. The letters indicate significant differences (no drying and 0 h: $F(1,8)=111.84$, $p<0.05$, 0 h and 24 h: $F(1,8)=16.10$, $p<0.05$, and no drying and 24 h: post-hoc Tukey HSD at a 0.05 level of significance, $F(1,8)=2.08$, $p=0.19$).

Total soluble sugar (TSS) levels significantly increased immediately after drying (0 h), compared with the statistically similar non-dried shoots (Fig 53). The TSS level was the highest in the experiment (93.34 ± 1.65 mg GFG.g^{-1} FWS) followed by the control that was dried (69.28 ± 3.31 mg GFG.g^{-1} FWS). After the dried shoots were transferred onto acclimation medium for 24 h, the TSS levels decreased and returned to pre-drying levels (Fig 53).

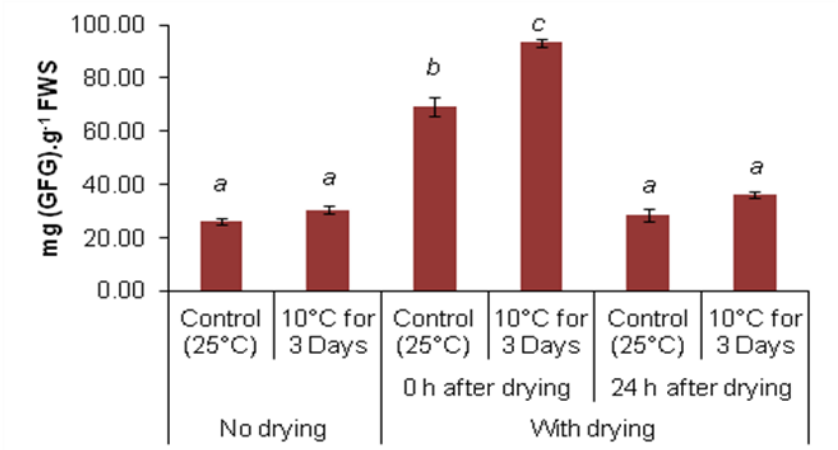


Fig 53. Total soluble sugar, GFG (TSS), levels of *in vitro E. grandis* shoots that were pre-treated at 10°C for 3 days and then dried for 80 min. Vertical bars represent mean ± SE. The letters indicate significant differences (no drying and 0 h: $F(1,8)=18.64$, $p<0.05$, 0 h and 24 h: $F(1,8)=15.96$, $p<0.05$, and no drying and 24 h: $F(1,8)=2.19$, $p=0.18$).

Consideration of both the starch and TSS together showed an interesting trend. Prior to drying TSS levels were 2-3 times higher than starch levels (Figs 52 and 53). Immediately after drying, the differences were more pronounced where TSS levels became several fold higher than starch levels, but after 24 h of recovery on acclimation medium the starch and TSS levels returned to the pre-drying trend (Figs 52 and 53).

6.2.2.2 Proline

Immediately after drying (0 h), the proline levels in the shoots were significantly higher than those in non-dried shoots (Fig 54). More specifically, the level in the control was higher (0.712 ± 0.015 mg proline/g FWS) than the level in shoots pre-treated at 10°C for 3 days (0.453 ± 0.020 mg proline/g FWS) (Fig 54). Indeed, both proline levels immediately after drying in shoots were approximately four-times greater than the respective levels of the non-dried shoots (Fig 54). After the dried shoots were recovered on acclimation medium for 24 h, the proline levels declined to levels similar to those of the pre-dried situation (Fig 54).

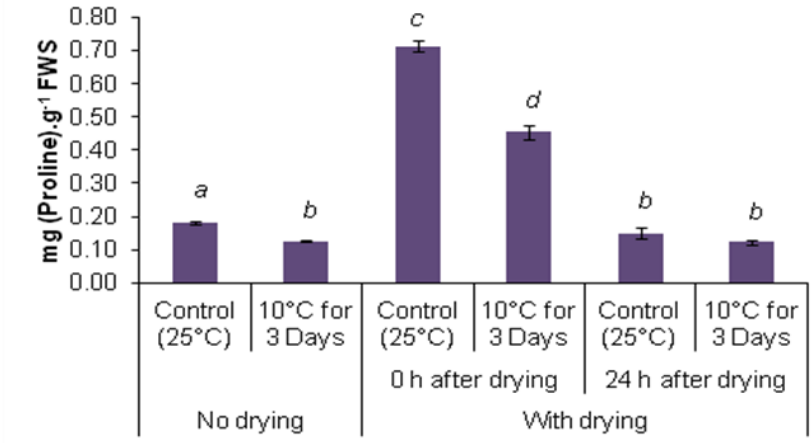


Fig 54. Proline levels of *in vitro E. grandis* shoots that were pre-treated at 10°C for 3 days and then dried for 80 min. Vertical bars represent mean ± SE. The letters indicate significant differences (no drying and 0 h: $F(1,8)=62.99$, $p<0.05$; 0 h and 24 h: $F(1,8)=55.80$, $p<0.05$; and no drying and 24 h: post-hoc Tukey HSD at a 0.05 level of significance, $F(1,8)=2.10$, $p=0.19$)

6.2.2.3 Phenolic acid and superoxide

The phenolic acid levels in the shoots showed a similar response to the other measure biomolecules (Figs 52-54). In this case, there was an increase in the phenolic acid levels in response to drying and then a decline during the recovery period (Fig 55). In all cases, shoots that had been pre-treated at 10°C for 3 days had higher levels of phenolic acid than the control, with exception of immediately after drying (Fig 55). It was interesting to note that during the recovery period, the phenolic acid levels dropped significantly lower than those measured prior to drying (Fig 55).

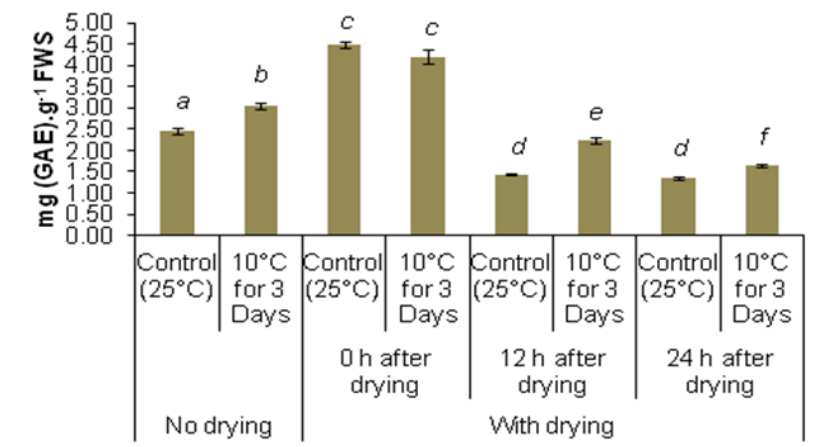


Fig 55. Phenolic acid levels of *in vitro E. grandis* shoots that were pre-treated at 10°C for 3 days and then dried for 80 min. Vertical bars represent mean ± SE. The letters indicate significant differences (no drying and 0 h: $F(1,8)=18.64$, $p<0.05$; 0 h, 12 h and 24 h: $F(2,12)=30.31$, $p<0.05$; and no drying and 24h: $F(1,8)=6.75$, $p<0.05$).

Immediately after drying (0 h), superoxide levels in the control increased significantly (Fig 56). Although the levels in shoots that were pre-treated at 10°C for 3 days increased numerically after drying, they remained similar to the levels of non-dried shoots (Fig 56). As seen with the other tested biomolecules, after the dried shoots were recovered on acclimation medium (tested at 12 h and 24 h), superoxide levels returned to pre-drying levels (Fig 56).

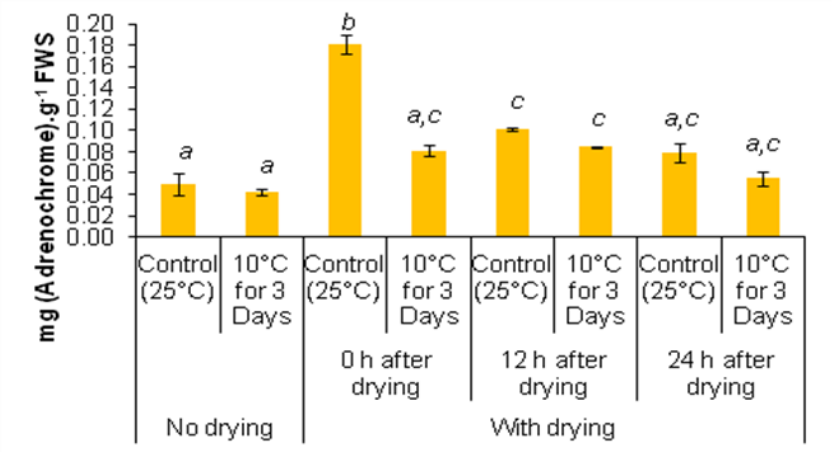


Fig 56. Superoxide levels of *in vitro E. grandis* shoots that were pre-treated at 10°C for 3 days and then dried for 80 min. Vertical bars represent mean ± SE. The letters indicate significant differences (no drying and 0 h: $F(1,8)=39.49$, $p<0.05$; 0 h, 12 h and 24 h: post-hoc Tukey HSD at a 0.05 level of significance, $F(1,8)=28.38$, $p<0.05$; and no drying and 24 h: $F(1,8)=1.18$, $p=0.31$).

Shoots that were not dried and those assayed immediately after drying showed that phenolic acid levels were higher than superoxide levels, with exception of the control immediately after drying where both levels appeared equal (Figs 55 and 56). After 12 h and 24 h recovery on acclimation medium, the superoxide level from the control that was dried was higher than its phenolic acid level. However, this trend was reversed in shoots pre-treated at 10°C for 3 days (Figs 55 and 56).

6.2.2.4 Proportions of biomolecules relative to the fresh weight of shoots (FWS)

A comparison of the proportions of each biomolecule showed that the roles of TSS, starch and phenolic acid were more prominent in shoots pre-treated at 10°C for 3 days than proline and superoxide, which were more closely associated with the control (Table 23). This was noted immediately after drying, where the TSS and phenolic acid proportions in the experiment (shoots pre-treated at 10°C for 3 days and then dried) were higher than the proportions of the control, whereas the superoxide, proline and the unknown component appeared higher in the control (Table 23). The unknown component proportions of both these treatments decreased immediately after drying, when compared with non-dried and recovered (24 h recovery on acclimation medium) shoots (Table 23). Starch proportions in non-dried and recovered experiment were higher than the controls (Table 23). Both the recovered control and experiment showed that phenolic acid proportions were less than 1% (Table 23), while proline and superoxide proportions were similar between the control and experiment (Table 23). In addition, superoxide proportions in recovered shoots were similar to non-dried shoots, whereas proline proportions were less than the non-dried shoots (Table 23).

Table 23. Overall proportion of each class of biomolecule within the dry mass of *in vitro E. grandis* shoots pre-treated at 10°C for 3 days and then dried for 80 min (with SE). Presented are the proportions in non-dried shoots and in shoots immediately after drying (0 h) and after 24 h on acclimation medium of the controls (25°C) and shoots pre-treated to 10°C for 3 days. The table represents the proportion (%) of biomolecules (mg biomolecules.g^{-1} fresh weight of shoots, FWS) relative to the mean dry mass of shoots (mg dry mass of shoots.g^{-1} FWS).

Proportion (%)	Without Cryopreparative Drying		With Cryopreparative Drying			
			0h After Drying		24h After Drying	
	Control (25°C)	10°C for 3 Days	Control (25°C)	10°C for 3 Days	Control (25°C)	10°C for 3 Days
TSS	17.59 ± 2.40	21.87 ± 1.90	47.65 ± 1.90	83.66 ± 1.48	12.98 ± 0.77	20.09 ± 0.67
Starch	3.85 ± 0.05	5.08 ± 0.53	0.55 ± 0.12	0.50 ± 0.04	3.86 ± 0.12	6.55 ± 0.46
Phenolic Acid	1.64 ± 0.05	2.18 ± 0.05	3.09 ± 0.05	3.77 ± 0.12	0.618 ± 0.015	0.918 ± 0.017
Superoxide	0.033 ± 0.007	0.030 ± 0.002	0.125 ± 0.006	0.072 ± 0.005	0.031 ± 0.004	0.031 ± 0.004
Proline	0.120 ± 0.003	0.090 ± 0.003	0.490 ± 0.011	0.406 ± 0.018	0.069 ± 0.008	0.069 ± 0.004
Unknown	76.76 ± 2.40	70.75 ± 0.58	48.10 ± 1.86	11.60 ± 1.36	82.44 ± 0.67	72.34 ± 0.21

6.3 DISCUSSION

6.3.1 Some physiological responses of *in vitro* shoots to cold acclimation at 10°C for 3 days, drying for 80 min over activate silica gel, and rehydration on acclimation or multiplication medium

Drying affected the physiological characteristics of *in vitro E. grandis* shoots (Table 22). The visible physical effects of drying were identical to those described in Section 5.3.2. Immediately after drying, the physical responses again included adaptive rolling or folding of leaves and visible reddish pigmentations, indicating the possible accumulation of anthocyanins and carotenoids in shoots (Table 22, and see Section 5.3.2.1). Following rehydration of these dried shoots, the majority of developed tissues and leaves underwent browning and senesced, whereas axillary buds survived and developed into light green shoots (Table 22). This again suggested that during drying shoots preserved regions of potential new growth such as axillary buds but sacrificed more developed photosynthetic leaves and tissue structures (see Section 5.3.2.1).

Despite the indistinguishable physical effects of drying, the pre-treatment of shoots at 10°C for 3 days (chilling or chilled-shoots) improved the physiological responses. One of the most significant improvements was reflected in the retention of 100% viability of dried shoots as a result of being pre-treated at 10°C for 3 days (Table 19). Chilling had improved the capabilities of the shoots to survive dehydration stress. The improvement could be attributed to underlying biochemical responses induced during chilling, which may have been carried through to the drying process, therefore promoting improved tolerance and viability (Fig 58).

6.3.1.1 Water content (WC) of pre-treated and dried *in vitro* shoots

The WC of shoots pre-treated at 10°C for 3 days decreased to 0.52 ± 0.05 g water.g^{-1} FWS, immediately after drying, and this was significantly greater than the dried control (Fig 49). The higher WC can possibly be attributed to the biochemical and osmotic adjustments in responses to the chilling and drying (Fig 58). Chilling has been shown to improve osmolyte accumulation in rooted-cuttings of *E. globulus* clones, which lowers osmotic potential resulting in leaf turgor potential becoming higher (Shvaleva *et al.*, 2008). In addition to osmotic adjustments, a further reduced stomatal conductance frequency may have also contributed in preventing water loss, since photosynthetic capacity of *in vitro* cultures are lower than plants in the natural environment (see Section 3.1). It was found that cold

treatment of wheat seedlings, for instance, also resulted in a lower stomatal frequency and higher osmolyte accumulation, which was suggested to have contributed to a significant reduction of water loss (Equiza *et al.*, 2001). Similarly, it was noted that WC level had increased after chilling in this study and this was also similarly seen with dried axillary buds (over activated silica gel) that were pre-treated with exogenous 5mg.l^{-1} ABA (0.47 ± 0.01g.g^{-1}) (Padyachee *et al.*, 2008). Such a similarity in WCs suggested that endogenous ABA could have been induced by chilling, thus contributing to the result of a high WC after drying (Chaves *et al.*, 2003; Shinozaki *et al.*, 2003; Shinozaki & Yamaguchi-Shinozaki, 2000). When dried shoots were rehydrated, the WC returned to levels similar to the non-dried shoots (Fig 49); this is also a common response in other tree species (Galle *et al.*, 2007; Kirschbaum, 1988; see Section 5.3.2.1). This implies that cellular and sub-cellular structures and the hydraulic processes of the shoots are capable of controlled rehydration and as such is an interesting area for further research (see Section 5.3.2.1). In this respect, Padayachee *et al.* (2008) showed that after 20 min of drying of isolated axillary buds, the cell wall and some membrane structures of *E. grandis* axillary buds remained intact, despite the damages incurred from drying.

Despite the WC of the chilled-shoots that were dried, the control WC after drying (0.32 ± 0.04 g water.g^{-1} FWS) is more suitable for cryopreservation purposes (Figs 49 and 58). However, by considering the hydraulic distribution of the whole shoot, the higher WC of shoots pre-treated at 10°C for 3 days could still be acceptable for cryopreservation. This is because the sub-cellular distribution of water in the cells and tissues of the shoots following drying could be specific to either a combination of inter-, intra- and extracellular regions (Fig 57). If water distribution is primarily located in extracellular regions such as the vascular tissue instead of intra- and intercellular localities, then the cells of the tissues could have the necessary vitreous cytoplasm suitable for cryopreservation (Fig 57). Such a vitreous state could be promoted by stress-induced biochemicals in response to the stressful effects of freezing (Figs 57 and 58). However, this must be confirmed on a sub-cellular level.

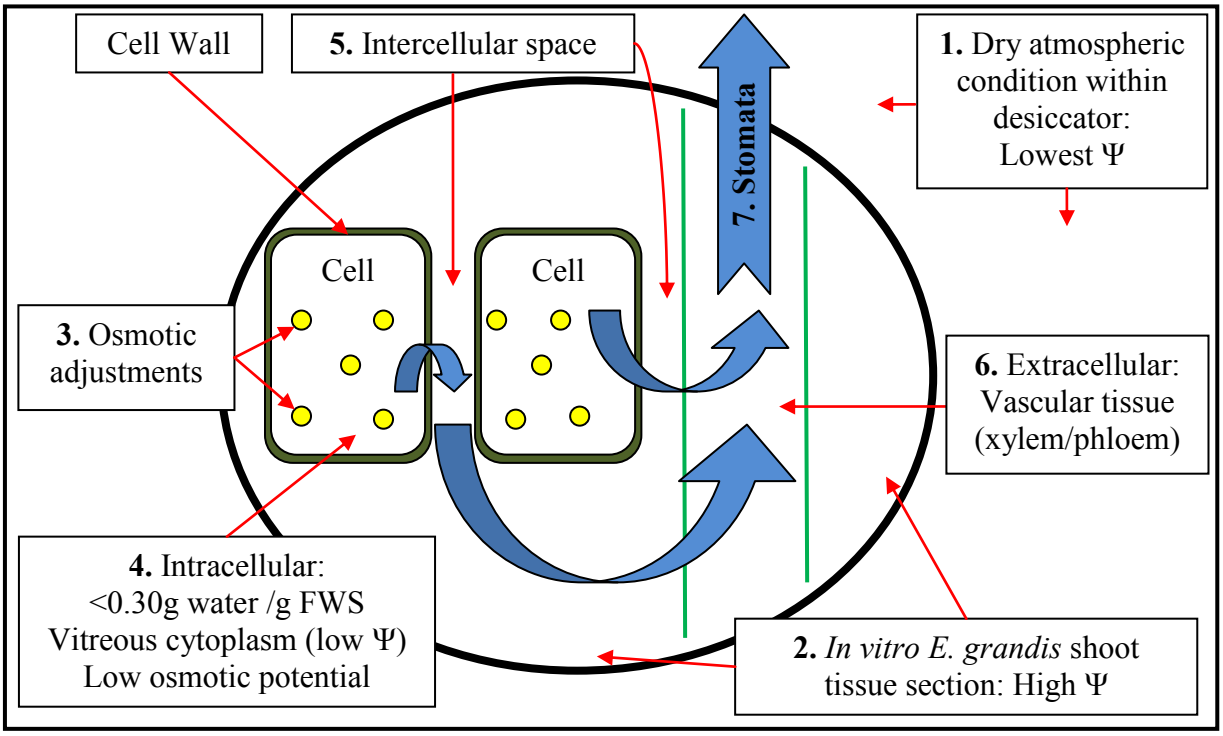


Fig 57. Schematic representation of the proposed distribution of water in cellular structures of *in vitro E. grandis* shoots pre-treated at 10°C for 3 days and then dried for 80 min. The numbers indicate the order in which to interpret the figure. (Ψ): Water potential. Blue arrows indicate the direction in which water moves within the cellular tissues of shoots.

6.3.1.2 Vigour of the pre-treated and dried *in vitro* shoots

Although viability was improved, the effects of drying reduced the vigour (emergence of axillary buds and growth of shoots) of recovering *in vitro E. grandis* shoots of both the control and treatment (shoots pre-treated at 10°C for 3 days and then dried) (Figs 50 and 51). Despite the consequences of drying, it was clear that the pre-treatment at 10°C for 3 days (chilling or chilled-shoots) had improved the vigour responses of the shoots that were dried and not dried (Figs 50 and 51). The improved vigour responses of non-dried shoots suggested that chilling may have induced adaptive mechanisms that initialized tolerance in shoots, which was further improved on by the responses triggered by drying (Fig 58). This improvement in survival by means of 'cross-tolerance' was also observed in other plant species as described in Section 6.1.

After recovery, the emergence of axillary buds and growth of shoots results were similar between the control and experiment (Fig 50 and 51) but the experiment showed an improved

viability (Table 20). This suggested that chilling and drying promoted the preservation of axillary buds in the shoots (see Sections 5.3.2.1 and 4.3.1). This strategy ensured that axillary buds of the experiment were protected and were better able to survive the negative effects of drying, thereby improving the likelihood of their viability and survival. Other sensitive tissue structures therefore (e.g. leaves and developing shoots) senesced despite the induced tolerance responses (Table 22). However, the extent of tissue browning was different between the recovered control and experiment that had been dried. After the 2-week recovery, signs of tissue browning were observed to a lesser extent in chilled-shoots than the control (Table 22). This further substantiated that chilled-shoots were better protected than the control, since stress-induced biochemical responses had continued from the chilling treatment. In addition, the formation of callus remained lower with drying than the non-dried shoots, which may indicate that oxidative stress was not severe enough to trigger callus formation (Gaspar *et al.*, 2002).

The vigour responses further supported the choice of pre-treating shoots at 10°C for 3 days (Figs 50, 51 and Table 22). Collectively the data infer the possibility of underlying mechanisms of 'cross-acclimation inducing cross-tolerance' in sub-tropical *E. grandis* (Fig 58). Some of these underlying mechanisms were biochemical responses induced during the chilling and subsequent drying treatments (Fig 58).

6.3.2 Some biochemical responses of *in vitro* shoots to cold acclimation at 10°C for 3 days, drying for 80 min over activate silica gel, and rehydration on acclimation medium

The effectiveness of chilling in inducing cross-tolerance in *E. grandis* shoots was clearly indicated through the drying stress-induced biochemical changes, which continued from the foundation established during the pre-treatment of shoots at 10°C for 3 days.

6.3.2.1 Starch and soluble carbohydrate responses

Drying of the shoots caused soluble sugars (carbohydrates) to increase, while the starch levels declined (Figs 52 and 53), similar to the description in Section 5.3.2.2.1. These responses further substantiated the importance of starch and carbohydrates and their metabolic relationship in imparting tolerance. The induced-metabolic activities may have been involved in converting starch into soluble carbohydrates, while also promoting the synthesis of stress-related soluble sugars that fulfilled their role in osmotic adjustment and as osmoprotectants in preventing further loss of water. Despite the commonality of the responses to drying, pre-

treatment of shoots at 10°C for 3 days (chilling or chilled-shoots) resulted in higher actual soluble sugar levels than the control immediately after drying (Figs 52 and 53). The chilling of shoots may have already initiated tolerance responses characterised as the 'maximum primary threshold' (Fig 58; and see Section 4.2.2.1), which resulted in improved starch and carbohydrates responses towards drying. This may suggest that the carbohydrate metabolic status of cold pre-treated shoots was 'primed' towards abiotic stress tolerance and thus responses towards drying were more immediate (Fig 58). This predominance of carbohydrate metabolism was also reflected in other plant species exposed to 'cross-tolerance' conditions (see Section 6.1).

When dried shoots were recovered on acclimation medium for 24h, soluble carbohydrates were converted back into starch (Figs 52 and 53). This trend of reversible carbon partitioning during recovery was also observed with drying alone (see Section 5.3.2.2.1). This re-conversion may have regulated the rehydration process of the cells and tissues (see Section 5.3.2.2.1), and thus may have minimized the extent of osmotic stress of dry tissue being placed onto tissue culture medium. The process of recovery returned the starch and TSS levels in shoots to those similar to the non-dried shoots (Figs 52 and 53), which suggests that the *E. grandis* shoots were metabolically capable of recovering starch and carbohydrate activities despite the effects of drying. This ability to recover was similarly demonstrated in water-stressed *E. pauciflora* seedlings, where recovery of carbon dioxide assimilation and stomatal conductance proceeded rapidly following the immediate recovery of leaf water potential (Kirschbaum, 1988). Although recovery responses were similar, the recovered starch level of the experiment was higher than the control and non-dried shoots (Fig 52). This may suggest that there were more soluble sugars synthesised during drying, in addition to the conversion from starch (Figs 52 and 53).

6.3.2.2 Proline responses

Drying induced high accumulation of proline within *in vitro E. grandis* shoots compared with the non-dried shoots (Fig 54). Despite the similarity of responses towards drying, it appeared as though that there was a stronger dependency of the control on proline for osmoprotection than in the experimental shoots because of the higher accumulated proline level with the control (Fig 54). This difference in proline dependency was also observed in soybean seedlings during different pre-dawn leaf water potentials (Riekert Van Heerden & Kruger, 2002; see Section 6.1). When the dried *in vitro E. grandis* shoots were recovered on

acclimation medium for 24h, the accumulated proline decreased to levels similar to the levels in chilled-shoots (Fig 54). This trend again substantiated the importance of rapid reduction of proline during recovery, because of its toxicity when present in excess (see Section 5.3.2.2.2). Furthermore, the involvement of proline in 'cross-tolerance' was also observed with other plants (see Section 6.1).

6.3.2.3 Phenolic acid and superoxide responses

High accumulation of phenolic acid was induced in both the control and experiment that were dried, compared with the levels of the non-dried shoots (Fig 55). This response resembled that to drying alone (see Section 5.3.2.2.3). It appears that the phenolic acid accumulation during the chilling treatment minimized superoxide to a tolerable control level (Figs 55, 56 and 58; and see Section 4.3.2.3). The chilling pre-treatment may have ensured that the phenylpropanoid pathways were already in a metabolic state of tolerance (Fig 58; and see Section 4.3.2.3). This metabolic state continued into drying, and further phenolic acid and anthocyanin accumulation were promoted (Fig 55), where their antioxidant role may have continued to minimize the superoxide to the tolerable level of non-dried shoots (Fig 56), and thus alleviating oxidative stress (see Section 4.3.2.3). Similarly, other plant species exposed to 'cross-tolerance' conditions have suggested higher phenolic acid accumulation (see Section 6.1).

On the other hand in the dried control, phenolic acid accumulation was accompanied by an equally high superoxide level (Figs 55, 56 and 58). The differences in phenolic acid and superoxide responses between the control and experiment may be related to the metabolic status and in particular the photosynthetic status of the shoots prior to drying. The capacity of metabolism might already have been further reduced in chilled-shoots (see Sections 1.4.3 and 4.3.2.3), whereas the capacity of the control was in a more active state of growth and development (Fig 58; and see Section 5.3.2.2.3). Thus, this would make the control shoots more susceptible to the effects of drying.

Differences in responses were also apparent between the control and experiment following 12h and 24h of recovery on acclimation medium, but the overall trend of recovery was similar to responses towards drying (see Section 5.3.2.2.3). The results suggested that the chilling pre-treatment ensured better tolerance responses from shoots, where the antioxidant capacity was more effective and long lasting at minimizing oxidative stress after drying and during recovery (Table 22 and Fig 56).

6.3.2.4 Indications from the contribution of biomolecules to the fresh weight of shoots (FWS)

Although the adaptive mechanisms employed were identical between the control and experiment (shoots pre-treated at 10°C for 3 days) after drying and during recovery, the resulting biochemical changes relative to its proportional contribution to the FWS differed. All the biomolecules showed re-allocations of proportions immediately after drying and during recovery, where soluble carbohydrates, starch and phenolic acid dominated over proline and superoxide (Table 23). This recurring trend of carbohydrate proportional dominance followed by the other biomolecules was also reflected in the previous chapters (see Chapters 3, 4 and 5). However, there was a difference with the proportional dominance of carbohydrates and unknown components between the control and experimental materials (Table 23).

The proportional differences in soluble carbohydrates could in part be the main factor why the viability of shoots after drying and recovery was 100% for the experiment, compared with the lowered viability of the control (Table 20). The metabolic state of the experimental materials may have been more appropriate for responding to the drying stress, whereas the control remained metabolically susceptible. Furthermore, the proportional dominance of soluble carbohydrates observed in this experiment was also similarly observed with 3-day cultured shoots that were only dried (see Section 5.3), but the proportional dominance of soluble carbohydrates under those circumstances was only able to ensure 86.7% viability (Table 16).

The recovery of dried shoots on acclimation medium for 24h showed that the proportions from the control and experiment reflected similarities to each of its non-dried counterparts (Table 23). The proportions after recovery may indicate that the experimental materials were more metabolically active than the control (Table 23), which indirectly implied that stress-tolerance responses induced during the chilling pre-treatment had indeed improved drying-stress tolerance and protection of shoots.

6.4 SUMMARY AND CONCLUSION

Regardless of the strategy employed in order to improve the tolerance responses of *in vitro E. grandis* shoots to drying, there were physiological impacts and injury brought about by drying that could not be prevented and/or avoided. Drying caused a low water potential environment which dehydrated the shoots, thus resulting in the substantial decrease of WC

within shoots (Fig 49). The dehydration presumably damaged and impacted cellular membrane systems that are integral to metabolic functions such as for photosynthesis and respiration, which subsequently resulted in leaf and tissue senescence and death (see Section 6.3.1), and the ultimate reduction of growth and development during recovery (see Section 6.3.1.2). Although there were unavoidable and unpreventable consequences of drying, underlying drying stress-induced biochemical responses, for the most part, were able to ensure the survival of shoots (88.9 ± 3.9%, Table 20). However, the employed strategy in this study of pre-treating shoots at 10°C for 3 days (chilling or chilled-shoots) before drying appeared to induce osmotic adjustments that improved the ability of the shoots to retain water (0.52 ± 0.05 g water.g^{-1} FWS, Fig 58), and osmoprotection that successfully ensured that subsequent viability was 100% (Table 20). These positive physiological responses can all be attributed to the interplay and combination of underlying biochemical responses that were induced by the initial chilling and subsequent drying conditions (Fig 58).

Although the underlying tolerance mechanisms employed in response to drying were the same between the control (directly dried) and experiment (shoots pre-treated at 10°C for 3 days and then dried), the biochemical response variations that brought about tolerance differed between the control and experiment (Fig 58). The initial control primary metabolic state was focused primarily on growth and development, and therefore may have been more susceptible to the damages and impacts caused by drying stress (Fig 58), which was evident in the physiological responses (see Section 6.3.1). Control biochemical responses imparting tolerance primarily depended on the equivalent contribution of soluble carbohydrates and other unknown responses, followed by high proline and phenolic acid levels (Fig 58). However, the diversified biochemical responses of the control towards drying was not effective in alleviating dehydration and secondary oxidative stress (represented by excessive superoxide levels), which ultimately could not ensure the viability of all the shoots (Fig 58 and Table 16).

On the other hand, chilling pre-treatment of shoots may have initiated abiotic stress tolerance-responses (Fig 58). These chilling-induced biochemical responses included higher accumulation of phenolic acid, the promotion of starch and carbohydrate metabolic activities at its 'maximum threshold' (see Section 4.3.2.1), and the reduced synthesis of proline, which together contributed in minimizing superoxide to tolerable levels (Fig 58). This implies that the metabolic state achieved by the chilling pre-treatment was the suppression of growth and development-related metabolism and a shift towards the promotion of stress-associated

metabolism, which could mean that less active metabolic systems would be susceptible to drying (Fig 58). In other words, the shoots were already acclimated to an abiotic stress, and when dried the transition of biochemical responses were more direct and focused on tolerance towards dehydration stress (Fig 58). These biochemical responses strongly indicate the vital role of soluble carbohydrates and starch in establishing tolerance in *E. grandis* shoots, followed by phenolic acid and to a lesser extent proline, which continued quenching superoxide to tolerable levels (Fig 58). The consequences of these biochemical responses ensured that the subsequent physiological responses were more positive than the control (Fig 50 and Table 21), even though the improvement could not prevent the inevitable damages and impacts incurred during drying.

Collectively, the results of this study confirmed that cross-tolerance can be achieved in sub-tropical *in vitro E. grandis* shoots. The pre-treatment of shoots at 10°C for 3 days successfully improved the tolerance responses towards drying (Fig 58). This process of achieving 'cross-tolerance' could be regarded as 'cross-acclimation', and would be an effective strategy in improving abiotic stress tolerance responses. 'Cross-acclimation' approaches could be applied in inducing 'cross-tolerance' responses towards the stressful process of cryopreservation. Despite the prospects of 'cross-acclimation inducing cross-tolerance' for the cryopreservation of *in vitro E. grandis* shoots, there are some uncertainties in regards to the biochemical responses when relating it to specific tissue organs of the plant and the ultra-structure of cells. There is a need to establish the hydraulic distribution of water within shoots, since the resulting WC achieved from 'cross-acclimation' may be too high for cryopreservation (see Section 6.3.1.2). By doing this, it would reveal whether the cytoplasmic state of cells has become viscous enough to achieve vitrification (see Section 6.3.1.2). In addition, it would be important to establish whether 'cross-tolerance' is transferred into axillary buds, since these meristematic tissues have been suggested to be a more suitable vegetative material in size and volume for cryopreservation (Padayachee *et al.*, 2009).

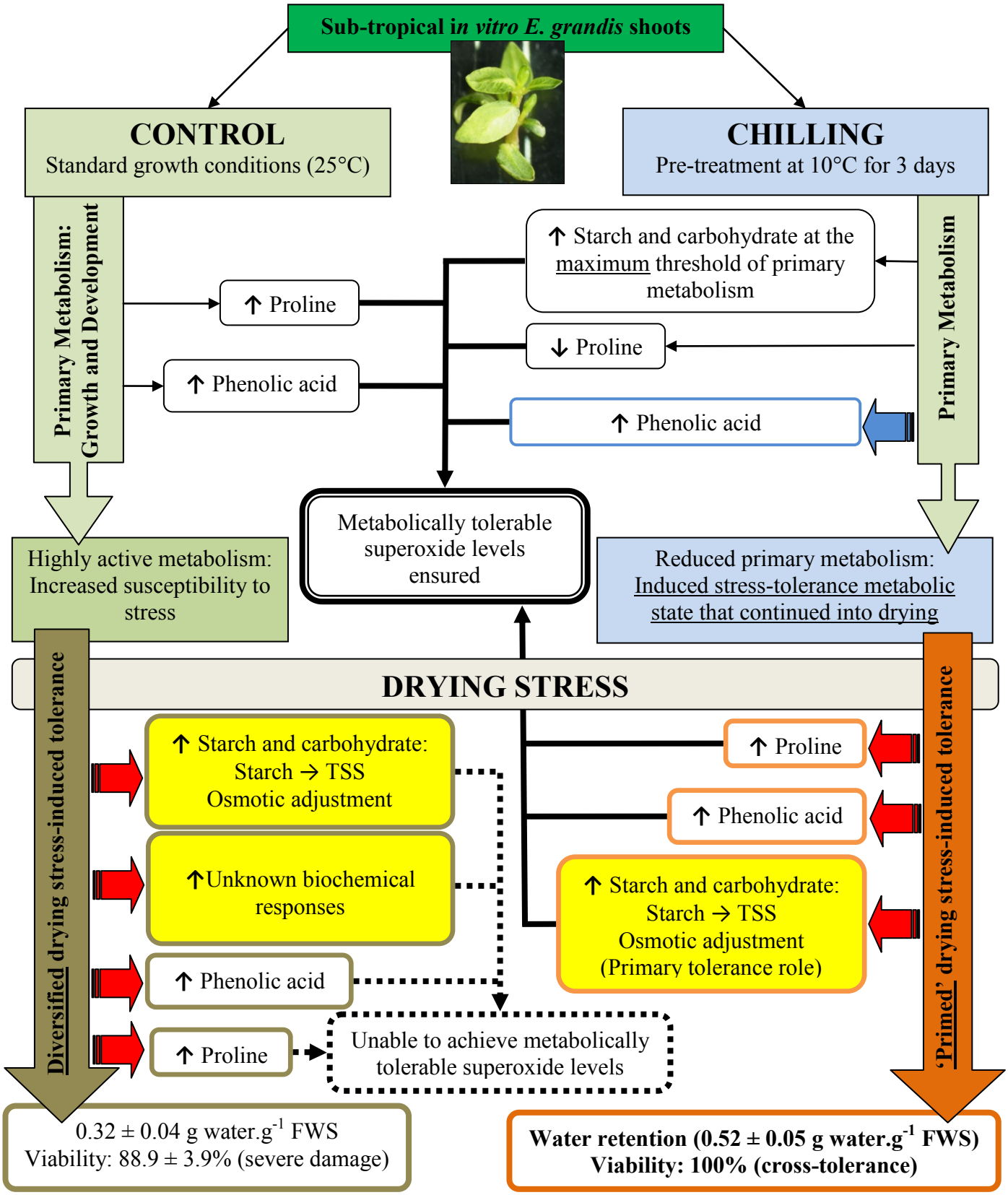


Fig 58. Schematic representation of drying tolerance and 'cross-tolerance' biochemical responses within *in vitro E. grandis* shoots. Thin black arrows indicate metabolic fluctuations during primary metabolism. Red and blue arrows indicate chilling- and drying-induced biochemical responses i.e. inducing additional and/or alternative pathways. Thick black and dashed arrows indicate the effect of these biochemical responses on superoxide levels. Increased (↑) and reduced (↓) metabolic activities in response to abiotic stresses. (→) Metabolic conversion of biomolecule. Yellow highlighted boxes indicate the dominant biochemical responses occurring during dehydration stress caused by drying.

CHAPTER 7. OVERALL CONCLUSION

7.1 Does pre-exposure to chilling stress induce cross-tolerance to cryopreparative drying of *in vitro* shoots of *E. grandis*?

The strategy of long term genetic conservation of *E. grandis* by means of cryopreservation is essential for tree improvement programmes, since it is an important and cost-effective approach for breeders to meet the evolving demands of commercial forestry (Padayachee *et al.*, 2009; Watt *et al.*, 2000ab). However, the stresses imposed by the cryopreservation procedures have limited the success achieved with vegetative *E. grandis* materials (Padayachee *et al.*, 2009; and see Sections 1.3-1.3.2). In particular, the cryopreparative pre-treatment by means of physical drying has been shown to reduce the viability and vigour of the *in vitro E. grandis* materials (e.g. axillary buds), even before reaching the later stages of the process (Padayachee *et al.*, 2009 and 2008). It is therefore important to ensure that materials remain viable after physical drying, in addition to achieving low water content for cryopreservation (Padayachee *et al.*, 2009 and 2008).

In this study, the potential of 'cross-acclimation inducing cross-tolerance' has proven to be an effective strategy of ensuring 100% survival of shoots after physical drying. The success of this strategy was dependent on the establishment of a fundamental appreciation of both the physiological and biochemical responses of the *in vitro E. grandis* shoots towards chilling, physical drying, and the consecutive treatment to both abiotic conditions. It appears that the 'cross-tolerance' was attributable to distinct patterns of biochemical responses in carbohydrates and starch, proline, and phenolic acid.

The chilling treatments (5°C, 10°C or 15°C for 1 or 3 days) alone neither affected viability nor water content, but were sufficient to strain the shoots so that biochemical fluctuations and adjustments were induced. These fluctuations and adjustments appeared to be specific to condition ranges and biochemical changes, although carbohydrate, starch, phenolic acid and some unknown components were observed to be the predominant biochemical responses for chilling. The culturing of shoots for 3 days at 5°C imposed the greatest strain as excess superoxide production could not be alleviated by the sustained proline, phenolic acid, and carbohydrate (including starch) metabolic adjustments. Treatment of shoots at 15°C promoted starch and soluble carbohydrate metabolic fluctuations, and promoted phenolic acid synthesis, while reducing proline content; and these responses may have been involved in the

maintenance of superoxide within tolerable/normal levels. The condition of 10°C for 3 days induced biochemical responses similar to shoots maintained at 15°C, but specifically triggered higher phenolic acid accumulation, which may have contributed to the positive vigour responses recorded on return of the material to growth room conditions. The positive physiological and biochemical responses from shoots treated at 10°C for 3 days suggested that this condition would be the most appropriate for 'cross-acclimation' for inducing 'cross-tolerance'.

The *in vitro E. grandis* shoots also responded at the biochemical level to physical drying alone for 80 min over activated silica gel. These imparted tolerance in shoots to dehydration stress, which primarily depended on the equivalent contribution of soluble carbohydrates and other unknown components. Despite the attainment of the favourable water content (0.32±0.04g water/g FWS) for cryopreservation, this diverse biochemical response ultimately was not effective in maintaining the viability of all shoots (88.9±3.9%). The shoots succumbed to the damages and injuries of dehydration stress and the excessive superoxide production that caused secondary oxidative stress.

The consecutive treatment of *in vitro E. grandis* shoots to the chilling pre-treatment followed by physical drying did not impact viability of the shoots (100%) and the material retained a higher water content (0.52±0.05g water/g FWS). The chilling pre-treatment stimulated osmoprotection and osmotic adjustments within shoots in response to the physical drying. These improvements strongly indicated the vital role of soluble carbohydrates and starch in effectively establishing tolerance for *in vitro E. grandis* shoots. In turn the phenolic acid and then proline components were also associated with alleviation of superoxide to tolerable levels. As a consequence of these biochemical responses, survival of the axillary buds was ensured and the physiological responses were more positive, despite the inevitable damages and injuries incurred during physical drying.

This study therefore clearly showed that 'cross-acclimation' by means of chilling pre-treatment at 10°C for 3 days could indeed induce 'cross-tolerance' towards physical drying in sub-tropical *in vitro E. grandis* shoots. Although 'cross-acclimation' and 'cross-tolerance' was evidenced by the distinct patterns of biochemical responses, these stress-associated biomolecules have been suggested to interact between each other in order to regulate abiotic stress tolerance responses.

7.2 Interaction between stress-associated biomolecules in conferring abiotic stress tolerance

The roles of individual stress-associated biomolecules in imparting abiotic stress tolerance have been well documented in the published literature. However, in addition to their specific roles, there is a growing body of evidence suggestive of their regulatory role in tolerance, where specific biomolecules interact with each other and along with ABA to regulate molecular, biochemical and physiological responses towards abiotic stresses. Soluble sugars/carbohydrates, ROS, antioxidants and proline are just some of the stress-associated biomolecules that are suggested to have some role in regulating tolerance.

Carbohydrate or sugar metabolism is a primary response to abiotic stress and is associated with osmoprotection and osmotic adjustments, but it can also modulate and regulate tolerance responses (Gupta & Kaur, 2005). End-products of carbohydrate metabolism have been noted to induce the expression of genes and proteins (i.e. protein kinases, phosphatases and calmodulin-binding protein), which regulate and modulate the signal transduction pathways of diverse processes such as photosynthesis, carbohydrate metabolism, oxidative stress defence and senescence (Gupta & Kaur, 2005; Steyn *et al.*, 2002). Soluble sugars are suggested, for example, to induce the synthesis of anthocyanins (Couee *et al.*, 2006; Steyn *et al.*, 2002), and also participates in the sensing and controlling of photosynthetic activity and its ROS balance (Couee *et al.*, 2006). It is also suggested that the degree of leaf-rolling or -folding in response to dehydration stress is positively correlated to the extent of soluble carbohydrate (or sugar) accumulation (Kadioglu & Terzi, 2007).

Sugars are integral partners in ABA signalling, since ABA has been suggested to be insufficient by itself to elicit responses under stress conditions (Verslues & Zhu, 2005). ABA together with high sugar accumulations are involved in enhancing proline accumulation in plants (Lehmann *et al.*, 2010; Ober & Sharp, 1994; Verslues & Zhu, 2005), regulating source-sink relations by negative feedback on photosynthetic gene expression, and simultaneously promoting starch biosynthesis (Rook *et al.*, 2006). Although proline accumulation in part can be influenced by other biomolecules (i.e. sugars), it has also been shown that proline metabolism can influence secondary metabolite synthesis (e.g. phenolic acid). In this regard, Shetty *et al.* (2002) suggested that higher proline synthesis could spontaneously increase the proline-linked pentose phosphate pathway, which then would subsequently support increased secondary metabolite synthesis in germinating *Vicia faba*. Furthermore, relatively small changes in ROS or even antioxidant counterparts are thought to

have effects on signal transduction and thus may influence downstream tolerance responses (Verslues & Zhu, 2005). For example, the enzymatic activity of chalcone synthase, involved in the synthesis of anthocyanins, can be induced by both soluble sugars and also ROS (Couee *et al.*, 2006; Steyn *et al.*, 2002).

In light of the above, it appears as though stress-associated biomolecules may be interdependent on one another in order to confer effective tolerance responses against abiotic stresses. Thus, the biochemical responses (soluble carbohydrates, starch, proline, phenolic acid, and superoxide) of *in vitro E. grandis* shoots might be functioning in synergy so that tolerance responses are able to ensure the viability of all shoots that experienced chilling and cryopreparative drying.

7.3 Future recommendations

In terms of cryopreservation, the prospects of employing 'cross-acclimation' to induce 'cross-tolerance' from *in vitro E. grandis* and even from other *Eucalyptus* materials towards surviving cryopreservation are possible. It is therefore necessary to progress to the next stages of cryopreservation, in order to determine whether 'cross-tolerance' can ensure subsequent viability of vegetative materials throughout the whole cryoprocedure. However, because of their smaller size, volume and lower tissue complexity, isolated axillary buds are more suitable for cryopreservation than the presently used large and complex shoots. This constraint raises some important points that have to be considered before cryopreservation can proceed. Firstly, from a technical perspective, one would need to determine whether axillary buds should be excised after the chilling pre-treatment and then dried or, alternatively, should axillary buds only be excised after both the chilling pre-treatment and then drying before cryopreservation. Secondly, from a biochemical perspective, it is unclear from this study whether the biochemical responses of 'cross-tolerance' were similarly shared in axillary buds as determined for the shoots. Thirdly, from a physiological perspective, the hydraulic distribution of water within the shoot structures should be established, since the WC of axillary buds was not investigated in this study. By doing this, it would reveal whether the WC and stress-associated biochemical profile of axillary buds would be suitable for cryopreservation.

Beside the prospects of 'cross-tolerance' for cryopreservation, there were also some biochemical and physiological questions that were raised by this study, which require further investigation. Padayachee *et al.* (2008) showed that *in vitro E. grandis* axillary buds pre-

treated with ABA and then dried for 20 min had a WC of 0.47 ± 0.01 $g.g^{-1}$, which was similar to the resulting WC of shoots (0.52 ± 0.05 $g.g^{-1}$) that were pre-treated at 10°C for 3 days and then dried for 80 min. Although the type of material and treatments employed differed between the study by Padayachee *et al.* (2008) and this study, the similar trend conveyed by the retention of water from the same species raises the possibility that there is an underlying relationship between endogenous ABA, chilling, and physical drying. It would therefore be necessary to investigate the physiological and biochemical effects of ABA and physical drying on shoots and chilling and physical drying on axillary buds to understand this relationship between treatments and type of material. Results of the biochemical and physiological responses, including WC, also suggest the possibility that the process of 'cross-tolerance' was in part also dependent on the metabolic and in particular the photosynthetic status of shoots, which would need to be investigated. Relative to biochemical responses, the predominance of soluble carbohydrates/sugars for tolerance is clearly confirmed by this study however, it would be interesting to distinguish the main types of soluble sugars involved in tolerance.

From a broader perspective, results of this study have also provided other possible research insights that would also further complement this investigation. These insights include: 1) How do the upstream signalling transduction pathways relate to downstream biochemical responses? 2) How do the biochemical responses towards the investigated abiotic stresses from different varieties of *Eucalyptus* species (e.g. cold and dehydration tolerant) compare with *E. grandis*? In addition, the concept of 'cross-tolerance' is based on the experience of plants in the natural environment, which include for the most part plants experiencing abiotic stresses in combinations. This therefore begs the question of whether the simultaneous pre-treatment of *in vitro E. grandis* shoots to chilling and physical drying could also induce distinct patterns of biochemical responses that would ensure survival, since 'cross-tolerance' was successfully induced.

CHAPTER 8. REFERENCES

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