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Causes of death in *Eucalyptus grandis* partially dried *in vitro* axillary buds

by

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A thesis Submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

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Johannesburg, 2014

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DECLARATION

I declare that this thesis is my own original work. It is being submitted for the Degree of Doctor of Philosophy to the University of the Witwatersrand, Johannesburg. It has not been submitted for any other higher degree or examination in any other university.

The experimental work described in this thesis was carried out in the School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Johannesburg, South Africa, under the supervision of Professor David Mycock (School of Animal, Plant and Environmental Sciences, University of the Witwatersrand) and Professor Paula Watt (School of Life Sciences, University of Kwa-Zulu Natal, Durban).

Signature: 

(Ida Masana Risenga)

Date: 23 May 2014

ACKNOWLEDGEMENTS

A major research project like this one is never the work of anyone alone. The contributions of many different people, in their different ways, have made this possible. I would like to express my heartfelt thanks and gratitude to the following:

The Almighty God, for the wisdom and perseverance that He has bestowed upon me during this research project, and indeed, throughout my life: “I have strength for all things in Christ who empowers me.” (Philippians 4:13), to God all be the glory!

My supervisor, Prof. D.J. Mycock, for his guidance, advice and patience throughout this research project. You created an environment of humour and in difficult times you were always there to let me know that everything was going to be all right. Thanks Dave!

My co-supervisor, Prof. M.P. Watt, for her support and encouragement from the initial to the final stage of this project. Your words boosted my courage and determination.

Sincere thanks are also extended to my colleagues: Prof. Richard Pienaar (my mentor), Dr Kershree Padayachee (my PhD coach), Mr Herman Du Preez and the EM unit staff members.

My special words of thanks are extended to the university of the Witwatersrand, Mellon foundation, National Research Foundation and Mondi Group SA for financial support.

Last but not least, I would like to thank my family and friends: Miriam and David Risenga (Mom and Dad), Pastor Martin and Tsakani Mukokie, Arthur Risenga, Matimba Risenga, Dr Cynthia Motsi, Katekani Mukokie (you’re the best!), Hitekani Mhlongo and Thomas Bigala.

CONTRIBUTIONS FROM THIS THESIS

PUBLICATION

Risenga IM, Watt MP, Mycock DJ. 2013. Programmed cell death and necrosis during cryopreparative drying of *in vitro Eucalyptus grandis* axillary buds. CryoLetters 34 (6), 583-597.

The paper was written by Ida Masana Risenga.

PAPERS PRESENTED

Risenga IM, Watt MP, Mycock DJ. 2010. Does cryo-preparative drying cause apoptotic-like programmed cell death or necrosis in isolated *Eucalyptus grandis* axillary buds? Cryobiology 61(3), 389-389. 47th Annual Meeting of the Society for Cryobiology, 17-20 July 2010. Bristol, United Kingdom.*

Risenga, I.M., Mycock, D.J., Watt, M.P. 2008. An ultrastructural and histological investigation on the effects of desiccation on isolated *in vitro* axillary buds of *Eucalyptus grandis*. Postgraduate Cross Faculty Symposium. 7-8 November. University of the Witwatersrand, Johannesburg, South Africa.

Risenga, I.M., Watt, M.P., Edwards,N., Mycock, D.J. A histological examination of desiccated *Eucalyptus in vitro* axillary buds. South Africa Journal Of Botany, Volume 74, Issue 2, April 2008, page 337. South African Association of Botanists Annual Conference. January. Drakensville, South Africa.

POSTER PRESENTED

Risenga, I.M., Padayachee, K., Watt, M.P., Edwards, N., Mycock, D.J. 2009. An ultrastructural and histological examination of desiccated *in vitro* axillary buds of *Eucalyptus grandis*. Plant Biotic Stress Tolerance. International Conference. 8-11 February 2009. Vienna, Austria.

*Presentation award: Peter L. Steponkus Crystal award – best student presentation

ABSTRACT

Eucalyptus grandis and its hybrids is the most important and widely planted eucalypt in South Africa. It has a wide range of uses including pulpwood, poles, firewood, charcoal, flooring, mining, furniture and general carpentry. Conservation of plant genetic resources including those used in agriculture, horticulture and forestry has become an issue of common global concern. Cryopreservation involves the storage of plant material at ultra low temperature (-196°C). The techniques for cryopreservation currently in use are varied and include the older classical techniques and the new vitrification-based techniques. Storage of biological material at -196°C causes metabolic functions to slow down considerably and minimize biological degradation, thus allowing for long-term preservation. However, there are particular stresses associated with the freezing process, e.g., ice crystal formation and cryo-dehydration, which may severely damage the material. Tolerance to drying is the key to successful cryopreservation and is commonly used in the preparation of *in vitro* material for cryostorage. However, drying may result in damages and a number of stresses that may activate caspase-like proteases and trigger cell death processes such as programmed cell death and necrosis. During the drying process, the physical and physiological characteristic of the cell changes because of the removal of water and damage is reflected by the lack of resumption of normal activity upon rehydration.

As part of a cryo-procedure, *Eucalyptus grandis* axillary buds isolated from *in vitro* shoots were dried over silica gel for 20 minutes. Pre-treatment of the shoots with 5mg.l^{-1} ABA for 5 days resulted in partial resistance of the isolated buds to water loss (76% to 45%) as compared with untreated buds (76% to 33%). Concomitantly, viability decreased from 100 to 70% for ABA treated buds and to 55% for the untreated buds. Ultrastructural examination showed cellular responses to drying, ranging from cell death, through partial disruption to organelles to apparently normal ultrastructure. The use of the vital stains, 4,6-diamidino-2-phenylindole and propidium iodide, showed that certain regions of the buds (e.g. the leaf primordia) were the most prone to drying damage. The meristem, however, appeared to survive drying and for up to 72 hours of rehydration.

High Reactive Oxygen Species (ROS) activity was associated with bud excision and the drying procedure. Caspase-3-like protease activity was detected after drying and rehydration in both nonviable treated and untreated buds, but not in the hydrated controls. The Caspase-3 inhibitors Ac-DEVD-CHO, pepstatin and leupeptin partially suppressed that activity. The ultrastructural studies and the use of the vital stains provided confirmation of the beneficial effects of ABA. The detection of a caspase-3-like protease has provided some evidence that the rehydrated buds, that had ultimately died, had undergone programmed cell death. The ROS production during bud isolation which was exacerbated by the drying procedure is considered to be the trigger for the programmed cell death. Data in the present study showed the role of both necrosis and PCD in the death of the tissues of the axillary buds of *E. grandis* axillary buds. The data also contributed to the better understanding of the impact of cryoprocedures on these clonal tissues which are ideal propagules for forestry germplasm conservation.

SYMBOLS AND ABBREVIATIONS

ABA	-	abscisic acid
Ac-DEVD-AMC	-	acetyl-Asp-Glu-Val-Asp-amino methylcoumarin
Ac-DEVD-CHO	-	acetyl-Asp-Glu-Val-Asp-aldehyde
AL-PCD	-	apoptotic-like programmed cell death
BAP	-	benzylamino purine
DAPI	-	4,6-diamidino-2-phenylindole
HCl	-	hydrochloric acid
$g.l^{-1}$	-	grams per litre
mM	-	milliMolar
$mg.l^{-1}$	-	milligram per litre
MS	-	Murashige and Skoog (1962) nutrient medium
NAA	-	naphthylacetic acid
PCD	-	progagrammed cell death
PI	-	propidium iodide
ROS	-	reactive oxygen species
rpm	-	revolutions per minute
SEM	-	standard errors of the means
TEM	-	transmission electron microscope

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Background on *Eucalyptus* and its importance

The genus *Eucalyptus*, which belongs to the Martaceae family, is recognised as an economically important hardwood and extensively propagated throughout the world (Brooker, 2000). It includes over 700 species, which can occur in one of the three subgenera: *Symphomyrtus* (usually occurs in areas of high soil fertility), *Monocalyptus* and *Corymbia* (which grow in areas of low nutrient soils) (Brooker, 2000; Ladiges *et al*., 2003). There is tremendous diversity in the growth form of *Eucalyptus* species, ranging from straight-trunked trees to multiple-stemmed, shrubby mallees (Muralidharan and Mascarenhas, 1995; Ladiges *et al*., 2003).

Eucalyptus trees were discovered by a French botanist, Charles Louis L'Heritier de Brutelle, in Australia. They were popular as attractions in botanical gardens and regarded as botanical curiosities in Europe. In the nineteenth century, seeds were dispersed to other parts of the world by botanists, travellers, goldminers, traders, priests and soldiers (Turnbull, 1991). Since then, eucalypts have become one of the most widely planted hardwood species worldwide (Turnbull, 1991; Tournier *et al*., 2003). *Eucalyptus* trees grow under various altitudes and soil types, and are found in areas where the annual rainfall exceeds 100cm (Albaugh *et al*., 2013). Because of their remarkable growth rate, adaptability and useful products, eucalyptus are regarded as one of the most productive forest trees (Rockwood *et al*., 2008).

Eucalyptus trees are recognised for their significant contribution to the economies of countries such as Brazil, Columbia, Morocco, India, China, Chile, South Africa, Portugal and Spain (Rockwood *et al*., 2008; Stanturf *et al*., 2013). They are utilized for a wide range of products including veneer, plywood, lumber, fibreboard, high quality paper, mine props, poles, charcoal, firewood, honey, essential oils, tannin and landscape mulch. Eucalypt planting has increased in recent years. For instance, in tropical countries, rotations are as short as five years with yields as high as 70m^3/ha/yr (Rockwood *et al*., 2008). This is attributable to eucalypts' superior fibre, pulping properties and an increased demand for short-fibre pulp. Four eucalypt species and their hybrids from the subgenus *Symphomyrtus*, *Eucalyptus grandis*,

Eucalyptus urophylla, *Eucalyptus camaldulensis* and *Eucalyptus globulus*, constitute the majority (80%) of the eucalypt plantations worldwide (Rockwood *et al*., 2008).

In South Africa, there is approximately 515 000 hectares of *Eucalyptus* plantations (Albaugh *et al*., 2013). *Eucalyptus* trees were initially used predominantly for the production of mining timber, however, emphasis has shifted to pulp production. The wood produced by eucalypt plantations (young trees) is an excellent source of paper pulp because of the exceptional quality it imparts to writing and printing paper. Other uses include paperboard for packaging, poles for communication networks and dissolving pulp for the textile industry (Schulze, 1997; Pallett and Sale, 2004).

The most important and widely planted eucalypt in South Africa is *Eucalyptus grandis*. It is well suited for high quality paper production because of its fast growth rate and superior fibre properties (Arbuthnot, 2001). Therefore, it is the most commercially important *Eucalyptus* species in South Africa. *E. grandis* grows best in warm climates, where rainfall averages 1000 – 1200mm or more per year. In spite of its suitability to warm climates, it is sensitive to cold temperatures and drought, making it unsuitable for growth in areas prone to frost, snow and drought (Pallett and Sale, 2004). Hybridisation of *E. grandis* with other *Eucalyptus* species added considerable flexibility to the species. Examples include *E. grandis* x *E. urophylla*, *E. grandis* x *E. camaldulensis*, *E. grandis* x *E. tereticornis* (combining good growth with drought tolerance), and *E. grandis* x *E. nitens* (combining good growth with cold tolerance and rooting ability) (Pallett and Sale, 2004; Albaugh *et al*., 2013). Other advantages of hybrid development include greater disease resistance and improvement of wood properties for specific end products.

1.1.1 *Hybrid intensive forestry in South Africa*

Breeding of eucalyptus for industrial plantations forestry developed rapidly in South Africa (Bayley and Blakeway, 2002).Target traits include volume growth, wood density and pulp yield (Griffin, 2001).The tree improvement efforts focus on the use of hybrid and their clones (Eldridge *et al*., 1993). The breeding strategies that have been developed involve the identification and selection of superior (improved) quality trees suited to growth in a particular area (Bayley and Blakeway, 2002).This is then followed by a series of repeated crossings, establishment of progeny and selection, until a population of highly selected elite individuals is established (Eldridge *et al*.,

1993; Rockwood *et al*., 2008). Selection of elite species and hybrids is followed by mass propagation of such species through clonal propagation. The clonal propagation of the elite individual trees is achieved by establishing vegetative cuttings from the mature trees through coppicing, followed by macro and/or microcutting establishment in greenhouses and micropropagation (Watt *et al*., 1995, 1997). Such clonally propagated trees have been shown to retain superior characteristics, such as stem straightness, uniformity in the fibre and structure of the wood produced (Watt, 2014), which are required for commercial forestry. In addition to such 'conventional' practices, many forestry concerns make use of *in vitro* cultures, in particular axillary bud proliferation, for which that are many well-established protocols (Donald and Newton, 1991; Le Roux and van Staden, 1991; Watt *et al*., 2003; Jones and van Staden, 1994; Yasodha *et al*., 2004). Such *in vitro*-produced plants are then used as propagules for planting and/or to produce parent plants for clonal hedges to supply cuttings for commercial planting (Donald and Newton, 1991; Le Roux and van Staden, 1991; Watt *et al*., 2003; Jones and van Staden, 1994; de Assis *et al*., 2004; Alpoim *et al*., 2004). It is acknowledged, however, that extensive selection practices coupled with clonal propagation could result in erosion of genetic variability (Eldridge *et al*., 1993). This would increase the vulnerability of the plantation to changing environmental conditions, pathogens and compromising any long-term gains. Therefore, breeders must maintain a balance between maximizing genetic gain for improved productivity and minimizing genetic erosion. This is achieved by employing a strategy that involves the maintenance of three populations: a population with a broad genetic base, a breeding population of moderately selected genotypes used in current breeding trials and an elite population of intensely selected trees for propagation (Watt *et al*., 2000a; White, 2001). Thus, the risk of complete plantation failure is buffered (Withers *et al*., 1990; Haines, 1994; Watt, 2014). In addition, to the need for maintenance of a broad genetic base, it is essential for tree improvement programmes to include mechanisms for maintaining useful progeny for periods commensurate with field-testing regimes.

Base collections are currently maintained as plantation stands, while active collections are maintained as clonal hedges established from rooted cuttings of selected genotypes or rooted cuttings in greenhouses (Watt *et al*., 2000a), and/or

hydroponic systems (Alpoim *et al*., 2004; Li and Pritchard, 2009) (Figure 1.1A-D). Such techniques are also used to maintain important clones during field testing periods. However, the development and maintenance of such systems is costly and labour-intensive. The establishment of plantation stands for conservation purposes is expensive in terms of land and water use (Watt *et al*., 1997, 2000a). In South Africa, the establishment of conservation stands is further constrained by the implementation of strict legislation regarding water use and the increasing unavailability of suitable land (Ford-Lloyd and Jackson, 1991; Watt *et al*., 1997; Dye, 2000). Hence, there is a need for the development of alternative methods of maintaining base collections that are less dependent on land utilisation. Further, the maintenance of important genotypes in clonal hedges and hydroponic systems render such genotypes vulnerable to bacterial, viral, fungal pathogens and pests (Li and Pritchard, 2009; Kaviani, 2011; Sharma *et al*., 2013). Although greenhouse and field-based methods of maintaining germplasm are important components of commercial breeding, technologies such as *in vitro* storage, that could minimize such risks, should be included in conservation strategies (Watt *et al*., 1997, 2000a, 2000b; Rao, 2004; Benson, 2008; Kaviani, 2011).

1.2 Plant germplasm conservation

The storage of plant cells, tissues and organs under physical and chemical conditions *in vitro* has been established as a reliable means of conserving vegetatively propagated plant material (Figure 1.1E-F) (Rao, 2004; Reed, 2008; Kaviani, 2011). The use of *in vitro* techniques is significant because of the numerous advantages associated with the technology (Cruz-Cruz *et al*., 2013). For example, maintenance of cultures under aseptic conditions ensures that the material is free from fungi, bacteria and viruses (Reed, 2008; Rao, 2004; Cruz-Cruz *et al*., 2013). The risk of loss of useful germplasm is substantially minimized as compared with greenhouse and field-based conservation. In addition, the requirement for large areas of land for the establishment of conservation stands and costs of maintaining genotypes in clonal hedges and greenhouses are reduced (Rao, 2004; kaviani, 2011; Cruz-Cruz *et al*., 2013). Furthermore, increasing interest in the application of genetic engineering in agriculture, horticulture and commercial forestry has emphasized the necessity for efficient storage techniques (Cruz-Cruz *et al*., 2013; Blakesley and Kiernan, 2001; Kaviani, 2011; Wang *et al*., 2012; Panattoni *et al*.,

2013). Transgenic lines necessitate specialised facilities, extensive monitoring and field assessment, as with traditional breeding methods, evaluation of the traits of interest may take many years (Blakesley and Kiernan, 2001). In this respect, *in vitro* storage offers a relatively low cost storage option.

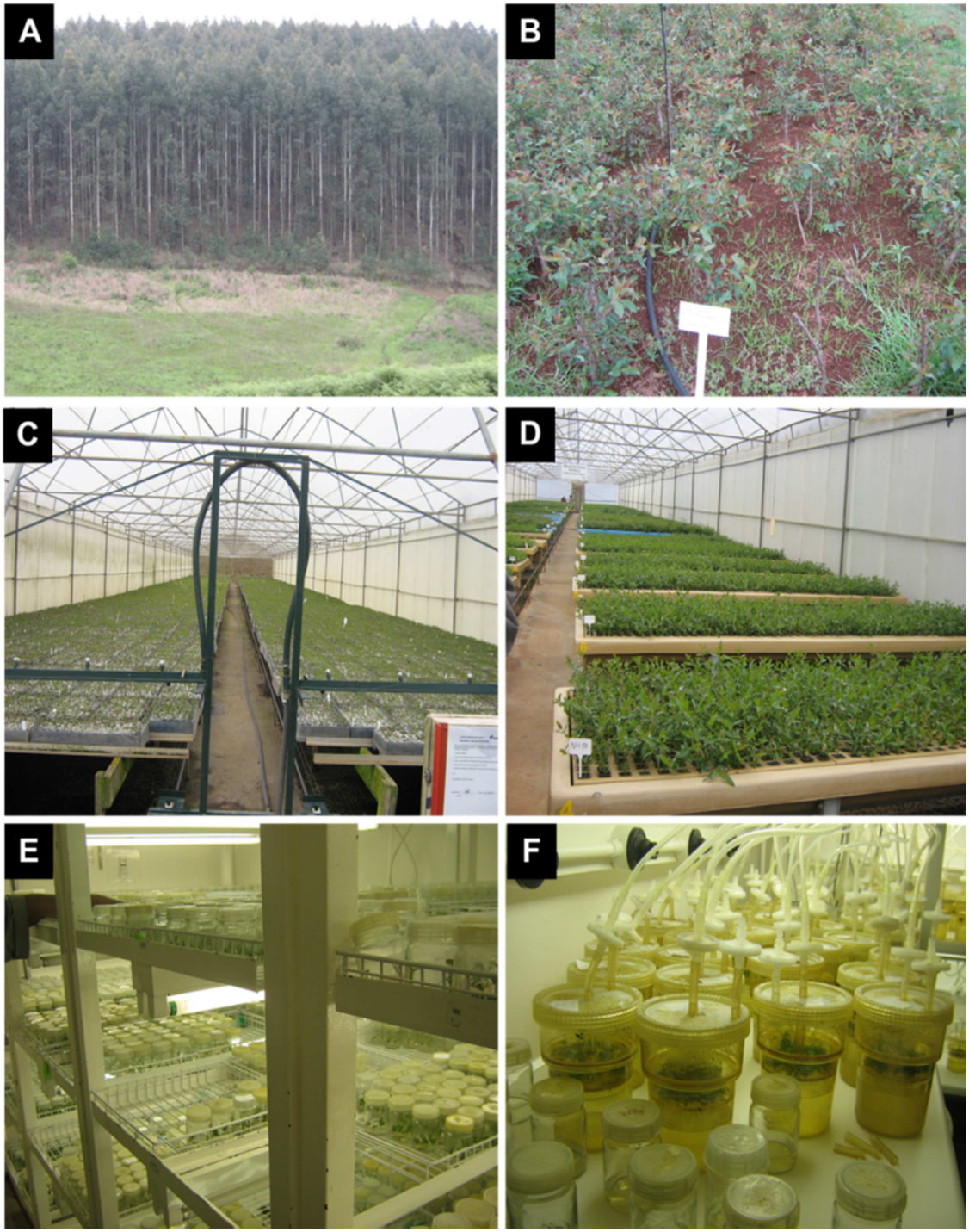


Figure 1.1 Examples of techniques of propagating *Eucalyptus* that are currently employed by Mondi Group, KwaZulu Natal, South Africa: (A) Field plantations, mature trees from which seeds can be collected. (B) Clonal hedges. (C) Cuttings maintained in the greenhouse. (D) Cuttings grown in hydroponic systems. (E-F) *In vitro* shoots cultures.

In vitro storage may be broadly divided into: short-medium or long-term. Short-medium-term storage involves the reduction of growth of plant material (minimal growth), thus allowing for storage for a few months to a few years (reviewed by Rao, 2004; Kaviani, 2011; Engelmann, 2011). Methods which are used for medium-term storage include reduction of temperature and light intensity (Withers and Englemann, 1997; Normah *et al*., 2011), addition of osmotic growth inhibitors such as mannitol to the culture medium (Engelmann, 2011), addition of growth inhibitors such as abscisic acid, or alterations of gaseous conditions in the culture vessel (Paunesca, 2009).

Despite the many advantages of medium-term storage, the methods are labour-intensive and there is always the risk of losing material through contamination or human error (Abreu-Tarazi *et al*., 2010; Senula and Keller, 2011). Moreover, *in vitro* cultures of some species such as plant zygotic, callus and somatic embryos are subject to somaclonal variation (the spontaneous development of mutations in *in vitro* cultures) (Keller, 2005; Kaviani, 2011). This is undesirable particularly when the maintenance of true-to-type clonal material is the primary goal. Although medium-term storage can be used to achieve one of the basic prerequisites of any germplasm conservation strategy, *i.e.* the ability to regenerate genetically stable plants (Rao, 2004), it is not ideal for the long-term maintenance of valuable germplasm (Abreu-Tarazi *et al*., 2010).

1.1.2 Cryopreservation

Cryopreservation is based on the storage of biological material at subzero temperatures, often in or just above liquid nitrogen (-196°C) (Reed, 2011; Berjak *et al*., 2011a; Engelmann, 2011, 2012; Cruz-Cruz *et al*., 2013; Uchendu *et al*., 2013). Cryopreservation is now recognized as a practical, efficient and economical tool for long-term storage of vegetatively propagated plant germplasm (Reed, 2008; Li and Pritchard, 2009; Engelmann, 2011; Cruz-Cruz *et al*., 2013). It offers several advantages such as storage of cultures in small quantities, minimal maintenance and protection from contamination (Benson, 2008, 2011; Gonzalez-Arnao *et al*., 2009; Uchendu *et al*., 2013). Moreover, the plant material can be stored in a stable state for a theoretically infinite period. This is because at subzero temperatures cell division and biological activities are significantly reduced until tissue revival (Engelmann, 2004, 2011, 2012).

Over the last 25 years remarkable progress has been made in this field; more than 200 different plant species have been successfully cryopreserved in various forms (Reed, 2008; Engelmann, 2011, 2012), and the number of cases is increasing. In all plant cryopreservation techniques the regrowth of propagules is the most important factor (Reed, 2011; Wang *et al*., 2012) that is recovery of viable material is the ultimate goal of cryopreservation (Reed, 2008; Benson, 2008; Engelmann, 2011, 2012; Wang *et al*., 2012). In the applicable techniques of cryopreservation, the induction of tolerance to drying in the tissues is an essential step prior to the immersion of the material in liquid nitrogen (Engelmann, 2010, 2011, 2012; Benson and Harding, 2012). Water status and cryoprotective strategies are the influential determinants for survival (Rabba'a *et al*., 2012). Reduction of water content to a level which retains viability yet is low enough to prevent ice crystal formation is an essential step for successful cryopreservation (Engelmann, 2012). Some plant materials such as orthodox seeds (Berjak, 2006; Berjak *et al*., 2007; Berjak and Pammenter, 2008) and dormant buds (Volk *et al*., 2008; Jenderek *et al*., 2012) can withstand extreme dehydration. However, in most cases plant tissues such as calli (Baghdadi *et al*., 2011), somatic and zygotic embryos (Suprasanna *et al*., 2008; Ibrahim *et al*., 2012), axillary buds (Padayachee *et al*., 2008, 2009) and shoot tips (Sharaf *et al*., 2012) contain high amounts of cellular water and are extremely sensitive to water loss. Since most of these tissues and organs are not inherently tolerant to drying, it is necessary to improve tolerance to drying to protect them from severe drying injury (Suzuki *et al*., 2006; Kaviani *et al*., 2012; Beck *et al*., 2010).

Cryopreservation techniques have been categorised into 'the classical' and 'the new vitrification'-based methods. Classical techniques are based on freeze-induced dehydration, while the new methods are based on vitrification (Engelmann, 2004, 2010, 2011, 2012). Classical techniques are not effective for low temperature sensitive species such as tropical species due to their inability to survive low and freezing temperatures (Berjak *et al*., 2011b). Furthermore, these techniques require the use of expensive programmable freezing apparatus. The newer cryopreservation techniques are often vitrification-based procedures; where cell dehydration is performed prior to freezing by exposure of samples to concentrated cryoprotective solutions/media and /or desiccation (drying) (Reed, 2008; Engelmann, 2012). Cryopreservation and its associated preparative procedures result in the exposure of

the cryo-propagules to physical, chemical and physiological stresses which can cause injury (Benson *et al*., 2007). Nevertheless, the propagules can generally be successfully cryopreserved if intracellular ice crystal formation is avoided, since this causes irreversible damage to the constituent cells and tissues (Benson *et al*., 2006). In this regard, partial drying (or water removal) plays a significant role in preventing irreversible damage and maintaining post-thaw viability (Gonzalez-Arnao *et al*., 2008, Engelmann, 2010, 2012). The partial drying results in the concentration of the constituent biomolecules. During such drying, the physical and physiological characteristics of the organs/tissues and cells change because of the removal of the biologically important water. The loss of water from the cells results in an increase in viscosity that, as dehydration increases, moves toward the establishment of an amorphous matrix (Reed, 2008, Uchendu and Reed, 2009). This viscous state is useful in cryopreservation as it inhibits molecular rearrangement of water into a crystalline pattern which is detrimental to cellular structural integrity (Taylor *et al*., 2004).

Vitrification-based techniques are particularly suitable for cryopreservation of complex organs like plant embryos and shoot apices. It offers simplicity, it does not require a programmable freezer and is applicable to a wide range of clones, genotypes and species (Gonzalez-Arnao *et al*., 2009, Sakai and Engelmann, 2007; Engelmann, 2010).

1.3 The selection of *Eucalyptus grandis in vitro* axillary buds for cryostorage

One of the crucial factors that may influence the success of cryopreservation is the type of explant selected for storage (Panis and Lambardi, 2006; Paunesca, 2009; Reed, 2010). Cryopreservation of vegetative tissues such as axillary buds has enormous potential for long term preservation of clonally propagated species because of the minimal risk of somaclonal variation (Reed, 2010; Rabba'a *et al.*, 2012; Coste *et al.*, 2012; Kaity *et al.,* 2013). In addition, *in vitro* axillary bud proliferation offers the potential to regenerate a large number of explants from individual buds (Engelmann, 2000, 2010; Reed, 2010, 2011). As micropropagation via axillary buds is well established for *Eucalyptus* (e.g. Padayachee *et al*., 2007; de Assis *et al*., 2004; Watt *et al*., 2004, 2014), it was envisaged that cryopreservation of axillary buds may be useful in this regard. By using such material, the risk of

somaclonal variation is reduced even further since the buds may be regenerated directly (*i.e.* without an intervening callus stage) after cryostorage. However, the complete survival of cryopreserved explants is generally difficult to achieve (Takagi, 2000; Yamamoto *et al*., 2012) and this is particularly so for *Eucalyptus* clones and species of importance to the South African forestry industry (Padayachee, 2007)

In order to maintain high productivity and meet future breeding challenges, the South African commercial forestry industry thus requires a strategy to maintain important genotypes during field testing and maintain a broad genetic base to complement current preservation techniques.

1.4 Cryo-preparative drying of *Eucalyptus grandis in vitro* axillary buds

Physical drying is one of the methods that is used for preparing plant material for storage at -196ºC (Gonzalez-Arnao *et al*., 2008, 2009; Engelmann, 2010). The process of drying involves the loss of most of the protoplasmic 'free or bulk' water and the survival of the material with only the 'bound' water, which is associated with the cellular constituents and cytomatrix. The danger of protein denaturing and membrane fusion also increases (Caramelo and Iusem, 2009). Damage that occurs during the drying process is only manifested when the cells rehydrate, furthermore, rehydration itself can cause damage and stress (Uchendu and Reed, 2008; Cruz-Cruz *et al*., 2013). If cells do not have any protective mechanisms against the changes caused by drying, then, during rehydration the cell will not be able to resume normal activity (Uchendu and Reed, 2008, 2009).

Various methods of preparing plant material to withstand lethal osmotic and evaporative drying stresses have been reported (Suzuki *et al*., 2006). Among these methods, a preculture on growth medium containing the plant growth regulator abscisic acid (ABA) is commonly used (Kumar *et al*., 2008; Zou *et al*., 2009; Rai *et al*., 2011a; Petijová *et al.*, 2012). ABA is a naturally occurring plant phytohormone and a major regulator of plant development and stress responses, including tolerance to dehydration (Böhmer and Schroeder, 2011). Under non-stressful conditions, ABA is at low levels in plant tissues (Kumar *et al*., 2008; Kakumanu *et al*., 2012). However, drying stress may induce the accumulation of endogenous ABA (Kakumanu *et al*., 2012). It has been established that ABA plays a role in water balance and cellular dehydration tolerance. In the former, the role in water balance is

mainly through regulation of guard cell activity (Jannat *et al*., 2011), whereas, the latter has to do with the ability to increase the biosynthesis of some of the cryoprotective solutes and induction of genes that encode various dehydration-tolerant proteins (Tuteja and Sopory 2008; Rai *et al*., 2011a; Petijová *et al*., 2012). Exogenous application of ABA has been used extensively in *in vitro* culture to induce dehydration tolerance. Evidence suggests that the adaptation and survival of plant tissues to drying stress may be enhanced by exogenous ABA (Stewart, 2001; Pospisilova *et al*., 2009; Ding *et al*., 2010; Rai *et al*., 2011b). Lu *et al.* (2009) reported increased survival abilities of *Ginkgo biloba* L. cell cultures after ABA preculture in comparison with sucrose treatment.

The success of cryopreservation is also dependent on the materials' level of tolerance to drying (Padayachee *et al*., 2008). *E. grandis* axillary buds require partial drying to protect them from damage caused by crystallization of intracellular water into ice during freezing. It was also established that the exogenous application of 5mg.l^{-1} abscisic acid (ABA) for a period of 5 days can induce tolerance to drying (Padayachee *et al*., 2008). The drying procedure for *E. grandis* axillary buds involved exposure to activated silica gel in an airtight desiccator for 20 minutes. Despite the ABA application, bud viability was negatively impacted by drying decreasing from 100 to 70% although this was significantly higher than non ABA treated material. Further, the ABA pre-treated buds showed a mosaic of intact cells, moderately damaged and severely damaged cells. These results raised the following key questions:

- What effect does partial drying have on the meristem of the axillary buds in terms of cellular integrity and nuclear morphology?
- Does axillary bud excision technique and partial drying trigger programmed cell death or necrosis?

1.5 Cell death

Cell death is a basic biological process that functions in many aspects of plant and animal development and in responses to stress (Gunawardena *et al*., 2007; Amirsadeghi *et al*., 2007; Jan *et al*., 2008; Lord and Gunawardena, 2012). Cell death may occur by necrosis, a non-physiological, destructive process involving disruption of membrane integrity and cell lysis, or by programmed cell death (PCD), an ordered and energy-dependent physiological process that is genetically programmed (Danon

et al., 2000; Reape and McCabe, 2008; Lord and Gunawardena, 2011). The necrotic pathway of death may be a result of severe trauma (necrosis), such as exposure to high or low levels of a toxin, heating, freezing, severe physical wounding and pathogen infection (Elmore, 2007; Kroemer *et al*., 2009; Kacprzyk *et al*., 2011).

In contrast, cells may die following the PCD pathway; either as part of a developmental program or as a response to environmental stresses (Kroemer *et al*., 2009). Various triggers, for example, heat shock, viral infection, protein synthesis inhibition, oxidative stress, hypoxia or nitric oxide, can induce both PCD and necrosis (Kroemer *et al*., 2009). It has been established that PCD and necrosis are two different processes although they can occur independently, sequentially, or simultaneously (Elmore, 2007). Also, it has been demonstrated that it is the stimuli or the degree of stimuli, which determines whether a cell will undergo necrosis or PCD. For instance at low doses a variety of stimuli, including hypoxia and heat shock induce PCD, however at high doses these same stimuli can result in necrotic cell death (McCabe *et al*., 1997; McCabe and Leaver, 2000; Evans, 2004; Elmore, 2007).

PCD was defined in 1972, on the basis of specific morphological changes occurring during a genetically controlled cell death initiated by a variety of environmental stimuli (Kerr *et al*., 1972). These cytological criteria were first described to discriminate a form of cell suicide from necrosis. It is only years later that some knowledge at the molecular level has been acquired. Each newly discovered step has been successively incorporated in a cell suicide pathway under the name of programmed cell death (McCabe *et al*., 1997; McCabe and Leaver, 2000). The term ‘programmed cell death’ was introduced in 1964, proposing that cell death during development is not of an accidental nature but follows a sequence of controlled steps leading to locally and temporally defined self-destruction (Lockshin and William, 1964). It is now well established that PCD is an intrinsic part of the life cycle of all multicellular organisms. PCD is characterized by dramatic morphological changes, particularly cytoplasmic membrane blebbing, cell shrinkage and chromatin condensation. Further, it is accompanied by fragmentation of the nuclear DNA to ensure genetic death, cytochrome *c* translocation from mitochondria to the cytosol, protein degradation by specific proteases (caspases) and disassembly of cells into

apoptotic bodies rapidly engulfed by phagocytes or neighbouring cells in animals (Widlak and Garrard, 2005; Jan *et al*., 2008; Conradt, 2009).

1.5.1 Programmed cell death in mammalian cells

PCD in animal systems is comparatively better known than in plants. Animal cells that undergo apoptotic cell death exhibit distinct morphological and molecular characteristics including changes in mitochondrial dynamics (Jones, 2000; Wang and Youle, 2009), influx of Na_2^+ resulting in plasma membrane depolarization and subsequent efflux of K^+ cations (Benítez-Rangel *et al*., 2011), up regulation of caspases (Denault and Salvesen, 2002; Potten and Wilson, 2004), actin cytoskeleton modulations (Franklin-Tong and Gourlay, 2008), apoptotic bodies formation, nuclear fragmentation, chromatin condensation and DNA laddering (Widlak and Garrard, 2005; Jan *et al*., 2008; Conradt, 2009).

B-cell CLL/lymphoma 2 (Bcl-2) family proteins are critical regulators for the PCD pathway in mammalian cells (Gross *et al*., 1999, Hengartner and Horvitz, 1994). Bcl-2 family proteins are either pro- or anti-apoptotic (Pepper and Bentley, 2000). For example pro-apoptotic Bcl-2 proteins include: Bcl-2 antagonist/killer-1 (Bak) and Bcl-2-associated X protein (Bax), while the anti-apoptotic include: Bcl-2-like 1 (Bcl-xL), Bcl-2-like 2 (Bcl-w), myeloid cell leukemia-1 (Mcl-1) and Bcl-2-related protein A1 (Bfl-1) (Potten and Wilson, 2004; Jan *et al*., 2008; Brunelle and Letai, 2009).Three general functions for Bcl-2 proteins (whether pro- or anti-apoptotic) have been recognised: a) dimerization with other Bcl-2 proteins; b) the interactions with proteins for mitochondrial homeostasis control, and c) ion channels or pores formation in the outer mitochondrial membrane (Pepper and Bentley, 2000; Brunelle and Letai, 2009). Most of the Bcl-2 proteins contain C-terminal hydrophobic transmembrane domains that cause them to be post-transcriptionally inserted into membranes such as the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear envelope (Hengartner, 2000; Danial and Korsmeyer, 2004; Potten and Wilson, 2004; Brunelle and Letai, 2009).

Mitochondria has a significant role in mammalian PCD. The PCD process is amplified by the release of apoptotic molecules from the mitochondrial intermembrane space (IMS) (Crompton, 1999; Jones 2000; Wang and Youle, 2009). Those molecules can exit the mitochondria through the mitochondrial permeability

transition pore (MPTP) and the pro-apoptotic pore forming Bcl-2 proteins, which may involve the voltage dependent anion channel (Crompton, 1999; Halestrap *et al*., 2000; Potten and Wilson, 2004; Kroemer *et al*., 2007; Billen *et al*., 2008; Wang and Youle, 2009).

The formation of MPTP in mammalian cells is generally a consequence of Ca_2^+ overload in the mitochondria (Crompton, 1999; Halestrap *et al*., 2000; Gunter *et al*., 2000). Under normal conditions Ca_2^+enters into the mitochondria though Ca_2^+ uniporter and exits though the exchange with either a Na^+ dependent or independent carrier (Gunter *et al*., 2000). Such movement results in a continuous cycling of Ca_2^+ across the inner membrane of the mitochondria. Oxidative stress result in an increase in free Ca_2^+ within the cell cytosol, which may subsequently cause a decrease in efflux versus influx of Ca_2^+ in and out of the mitochondria. Consequently, this may cause the formation of the MPTP (Crompton, 1999; Gunter *et al*,. 2000; Brunelle and Letai, 2009). Succeeding the MPTP formation, solutes flow into the mitochondrial matrix and cause a simultaneous drop in mitochondrial membrane potential. The disassociation of oxidative phosphorylation from the electron transport chain and the swelling of the matrix are thought to occur. The matrix swelling consequently disrupts the outer membrane of the mitochondria, thus causing it to be permeable or ruptured (Bernardi *et al*., 1999; Loeffler, 2000; Kuwana *et al*., 2005). Following the outer mitochondrial membrane permeabilization or rupture, apoptotic proteins such as cytochrome *c* (cyt-*c*) (Wang, 2001), apoptosis-inducing factor (AIF) (Susin *et al*., 1999) and endonuclease G (Endo G) (Li *et al*., 2001), Smac/Diablo (Verhagen *et al*., 2000) and Htr/Omi (Verhagen *et al*., 2002) are released from the IMS. Cytochrome *c*, a respiratory chain protein, binds with the apoptosis activating factor-1 (Apaf-1), once in the cytosol activating the apoptosome (Bratton and Salvesen, 2010). Apaf-1 contains a WD-40 repeat domain, of which may act as scaffolding for protein interactions, and is thought to be the cytochrome *c* binding site (Bernardi *et al*., 1999; Kroemer and Reed, 2000). Succeeding the apoptosome formation, it binds specific proteases to further the PCD process (Hengartner, 2000; Shi, 2001; Potten and Wilson, 2004; Kim *et al*., 2005; Bajt *et al*., 2006; Brunelle and Letai, 2009).

1.5.2 Programmed cell death in plant cells

Programmed cell death in plants can be either environmentally induced or developmentally regulated (Gunawardena, 2008; Solís *et al*., 2014). Environmentally induced PCD is an outcome of external biotic and abiotic factors and can be induced by such factors as heat shock (McCabe *et al*,. 1997; McCabe and Leaver, 2000), UV radiation (Danon and Gallois, 1998), pathogens (Mittler and Lam, 1997), low oxygen (Gunawardena *et al*., 2001), and density and salinity changes (McCabe *et al*., 1997). In contrast, developmentally regulated PCD occurs as part of normal, unperturbed development and presumably is a response to internal signals. Examples of developmentally regulated PCD include elimination of transitory organs and tissues (Browder and Iten, 1998), xylem differentiation (Fukuda *et al*., 1998), and leaf morphogenesis (Gunawardena *et al*., 2004, 2005).

Programmed cell death similarities between animal and plant cell death have been reported (Reape *et al*., 2008, Reape and McCabe, 2008; Zhang *et al*., 2009; Radziejwoski *et al*., 2011). Plant PCD includes a variety of types of cell death (Sanmartín *et al*., 2005, Li *et al*., 2008; Lord and Gunawardena, 2012). A number of hallmarks in animal PCD, such as DNA fragmentation (Reape and McCabe, 2008), cytochrome *c* release from mitochondria (Williams and Dickman, 2008), cell shrinkage (Zhang *et al*., 2009), chromatin condensation (Roa *et al*., 2009) and the activation or expression of various proteases, have been found in various plant systems (Reape and McCabe, 2010). However, despite the similarities, PCD pathways in plants are still not well understood (McCabe and Pennell, 1996; Gunawardena *et al*., 2004; Lord *et al*., 2011). In animal PCD (apoptosis), apoptotic bodies facilitate the engulfment of the cell committed to die by macrophages. The lack of cell wall is considered the reason for the non-persistence of cell corpses in animals unlike in plant tissues (Lord and Gunawardena, 2012). In plants, a number of cell types start functioning (e.g. tracheary elements) only after the cell death and the function is essentially facilitated by the persisting cell wall (Obara and Fukuda, 2004; Gunawardena, 2008; Lord and Gunawardena, 2012). Recently, van Doorn *et al* (2011) described a plant PCD classification based on morphological criteria. In that classification, plant cell death is divided into vacuolar cell death and necrosis. However, several examples such as the hypersensitive response and PCD of the cereal endosperm cannot be described by the use of these major classes (Lord and

Gunawardena, 2012). Although PCD pathways in plants are still less well-understood, research efforts are still being made to investigate factors that regulate PCD in plants. In this regard, Lord and Gunawardena (2012), have amalgamated evidence from literature that confirms that plants and animals do share some common characteristics in PCD.

1.6 Aim of this study

A cryo- protocol for isolated *E. grandis* axillary buds, although not optimal, has been devised (Padayachee *et al*., 2008). It includes a number of steps aimed at preserving viability after freezing, one of which involves drying of the material over activated silical gel for 20 minutes (Figure 1.2). In this study, application of 5mg.l^{-1} ABA for a preculture period of 5 days was used to induce tolerance to drying. This resulted in an induction of partial tolerance to drying but decreased viability from 100% to 70% survival. It was, therefore, hypothesized that the drying step may have triggered programmed cell death in the bud tissues. In line with this postulate the aim of this study was to investigate the causes of loss of viability of the axillary buds during the preparative drying step of the cryopreservation protocol.

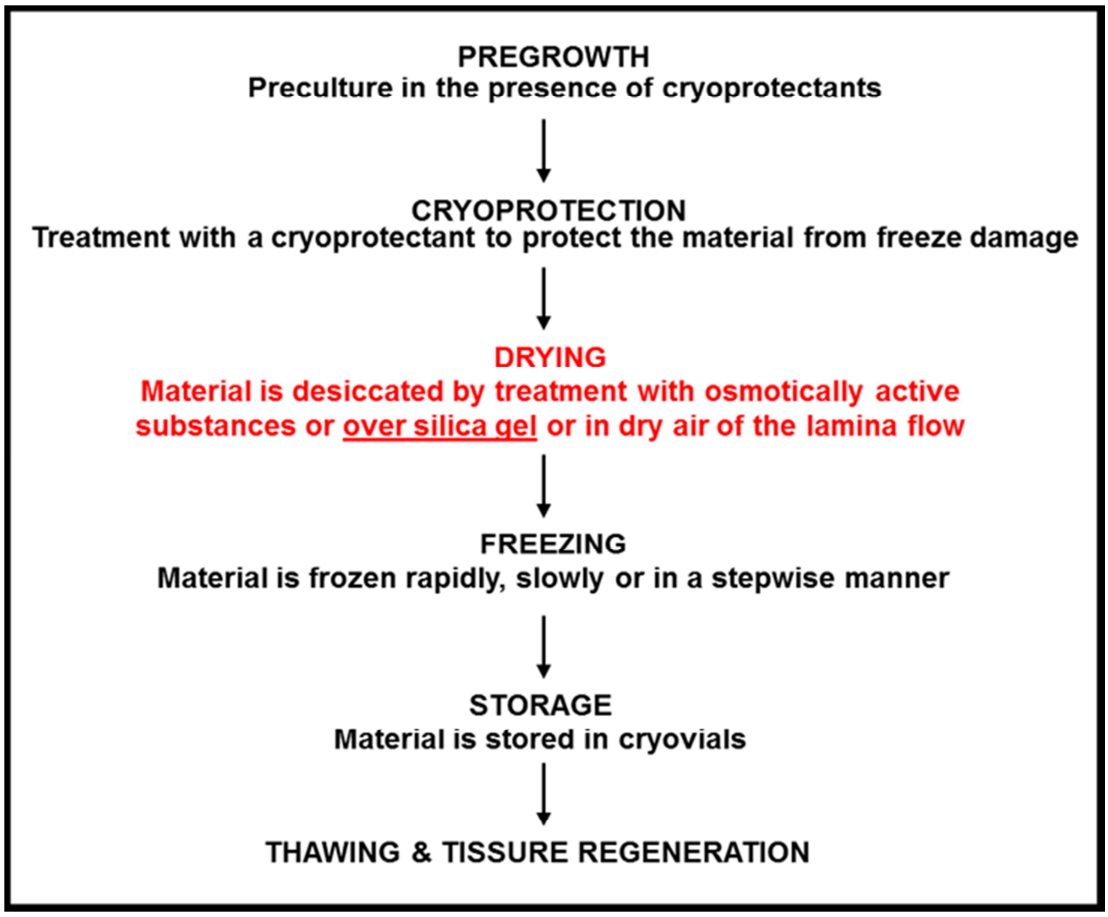


Figure 1.2. A schematic representation of a proposed protocol for cryopreservation of *Eucaluptus grandis in vitro* axillary buds (Padayachee *et al*., 2008).

CHAPTER 2: AN HISTOLOGICAL AND ULTRASTRUCTURAL EXAMINATION OF PARTIALLY DRIED *IN VITRO* AXILLARY BUDS OF *Eucalyptus grandis*

2.1 INTRODUCTION

Many plant species have been successfully cryopreserved through the development of various cryopreservation techniques (Reed, 2008; Engelmann, 2011; Kaczmarczyk *et al*., 2012). Since cryopreservation is a process that makes water unavailable for biological activities, most preparative methods are directed towards the attainment of tolerance or at least partial tolerance to drying. In vitrification-based procedures, tissue dehydration is performed prior to freezing (Sakai and Engelmann, 2007; Kaczmarczyk *et al*., 2008; Engelmann, 2011; Zhou *et al*., 2012). Vitrification of water in plant material is dependent on increased cellular viscosity, occurring as solutes in tissues become concentrated. Increased viscosity inhibits the coming together of water molecules to form ice (Reed, 2011). Cell viscosity enhancement is achieved using mainly the addition of cryoprotectants and water removal by evaporative drying or osmotic dehydration (Bilavcik *et al*., 2008; Benelli *et al*., 2012). In the case of drying methods where water is removed through osmotic dehydration and/or by evaporative means, there is however the additional problem of sensitivity to drying (Kaviani *et al*., 2012; Benelli *et al*., 2012).

It has been suggested that there are several problems which have to be overcome if plant tissues are to be tolerant to drying (Caboni *et al*., 2008; Varghese *et al.,* 2011). These include minimizing mechanical damage associated with the shrinkage of cells, maintaining the integrity of macromolecules and membranes by the accumulation of stress proteins, and minimizing free radical damage generated as metabolism becomes impaired (Deeba *et al*., 2012). In addition, upon rehydration such plant tissues must be able to repair damage caused by the drying and cellular expansion during re-absorption of water (Kaviani, 2010). However, most vegetative tissues are sensitive to drying processes which may result in the induction of severe chemical and mechanical stresses (Varghese *et al.,* 2011). Such stresses may lead to damage associated with turgor loss, rupture of the plasma membrane, oxidative stress and interruption of metabolic pathways (Kaviani, 2010). Furthermore, upon rehydration and expansion of the cells, cell membranes are in danger of rupturing.

Internal membranes such as the tonoplast (which separates the acidic content of the vacuole from the cytoplasm) and mitochondrial cristae may undergo changes which may not be easily reversed (Padayachee *et al*., 2008, 2009; Xing *et al*., 2010; Grigorova *et al*., 2012; Wang *et al*., 2012).

Plant tissues subjected to drying stress inevitably face a disruption of cellular homeostasis which affects the ultrastructure and functioning of subcellular organelles in particular the mitochondria (Atkin and Macherel, 2009; Blokhina and Fagerstedt, 2010). For instance, mitochondrial swelling and degraded cristae appearance was observed in drought-sensitive *Malus hupehensis* plants after 8 days of drought (Wang *et al*., 2012), and fewer mitochondria were observed in brown fine roots of *Fraxinus mandshurica* under drought stress, and the organelle inner membranes had disintegrated and disappeared to a certain degree (Xing *et al*., 2010).

In addition, plant tissues that are sensitive to water loss exhibit a wide range of responses to the stresses associated with cryopreparative drying (Rampino *et al*., 2006; Li *et al*., 2009). For example: 1) in embryonic axes and apical buds of *Camellia sinensis* L. cv.100, survival was not achieved even after pre-treatment with sucrose and dehydration (Kaviani *et al*., 2012); 2) in cherry plum shoot tips, a low 5 to 10% recovery of cryopreserved samples was achieved as the shoot tips were very sensitive to vitrification solutions (Vujovic *et al*., 2011); 3) in garlic shoot tips, an air-drying treatment, performed either before or after a vitrification solution treatment, was detrimental to the regeneration of cryopreserved shoot tips (Kim *et al*., 2004); and 4) the studies of Benelli *et al*., (2008) on *Populous* spp. germplasm showed that no survival was achieved with the encapsulation-vitrification technique.

Cryopreparative drying has the potential to cause stress that could increase ROS production. Evidence suggests that drying may lead to increased ROS production and subsequent oxidative stress (Roach *et al*., 2008; Lynch *et al*., 2011). In this regard, it has been shown that one mode of ABA action may be related to its role in the oxidative stress in plant cells (Mahmoodzadeh and Esparham, 2011). The antioxidant effect of ABA was reported in plant material such as *Begonia x erythrophylla* (Burritt, 2008).

Most plants can tolerate a certain amount of drying, but there is a limit after which viability is affected (Mycock, 1999). The water content at which the remaining water

in the plant tissues is freezable constitutes the “critical water” content (Suzuki *et al*., 2006; Engelmann, 2010). Drying below the “critical water” content results in severe damage and the ultimate loss of viability. As already mentioned, the basis for successful cryopreservation lies in the removal of cellular water prior to freezing in order to avoid the occurrence of ice crystals (Reed, 2008; Engelmann, 2010, 2011). Therefore, the understanding of the state of cellular water prior to, during, and after drying, as well as the consequences of its removal is essential in cryopreservation (Gonzalez-Arnao *et al*., 2008, 2009; Engelmann, 2010, 2011).

It has been established that the exogenous application of $5mg.l^{-1}$ abscisic acid (ABA) for a period of 5 days can induce some resistance to water loss in *E. grandis* axillary buds (Padayachee *et al*., 2008). This was demonstrated by the fact that when dried for 20 min, ABA pre-treated buds maintained significantly higher water content than untreated buds [$0.45g.g^{-1} \pm 0.02g.g^{-1}$(ABA pre-treated) and $0.33g.g^{-1} \pm 0.014g.g^{-1}$ (untreated)]. The exogenous application of ABA has been associated to protein synthesis and compatible solutes which play an important role in stress tolerance (Petijová *et al*., 2012). The external drying process, however, resulted in number of stresses. Therefore, this study was designed to contribute to the current understanding of the responses of *E. grandis* axillary buds to drying in terms of viability.

2.2 MATERIALS AND METHODS

2.2.1 Maintenance of parent plant, *in vitro* cultures and shoot multiplication

Eucalyptus grandis clonal cuttings were supplied by Mondi Business Group (Hilton, South Africa). The rooted cuttings (Figure 2.1) were maintained in the greenhouse (25ºC ± 10 ºC) at the University of the Witwatersrand, Johannesburg. Plants were watered daily and fertilizers were applied weekly, alternating between the following foliar fertilizers: $10ml.l^{-1}$ Trelmix® (Hurber [Pty] Ltd., Howick, SA), $0.3gl^{-1}$ Calmag® (Harvest Chemicals, Alrode, SA) and $10gl^{-1}$ Mondi Orange® (Harvest Chemicals, Alrode, SA). Systemic and foliar fungicides were also regularly applied: These include $2g.l^{-1}$ Dithane® (Efecto, SA), $1ml.\ l^{-1}$ Bravo® (Efecto, SA), $1g.l^{-1}$ Sporgon® (Bayer, Isando, SA) and $1.25ml.\ l^{-1}$ Folicur® (Bayer, Isando, SA). Plants were trimmed every four weeks to encourage shoot sprouting/coppicing.

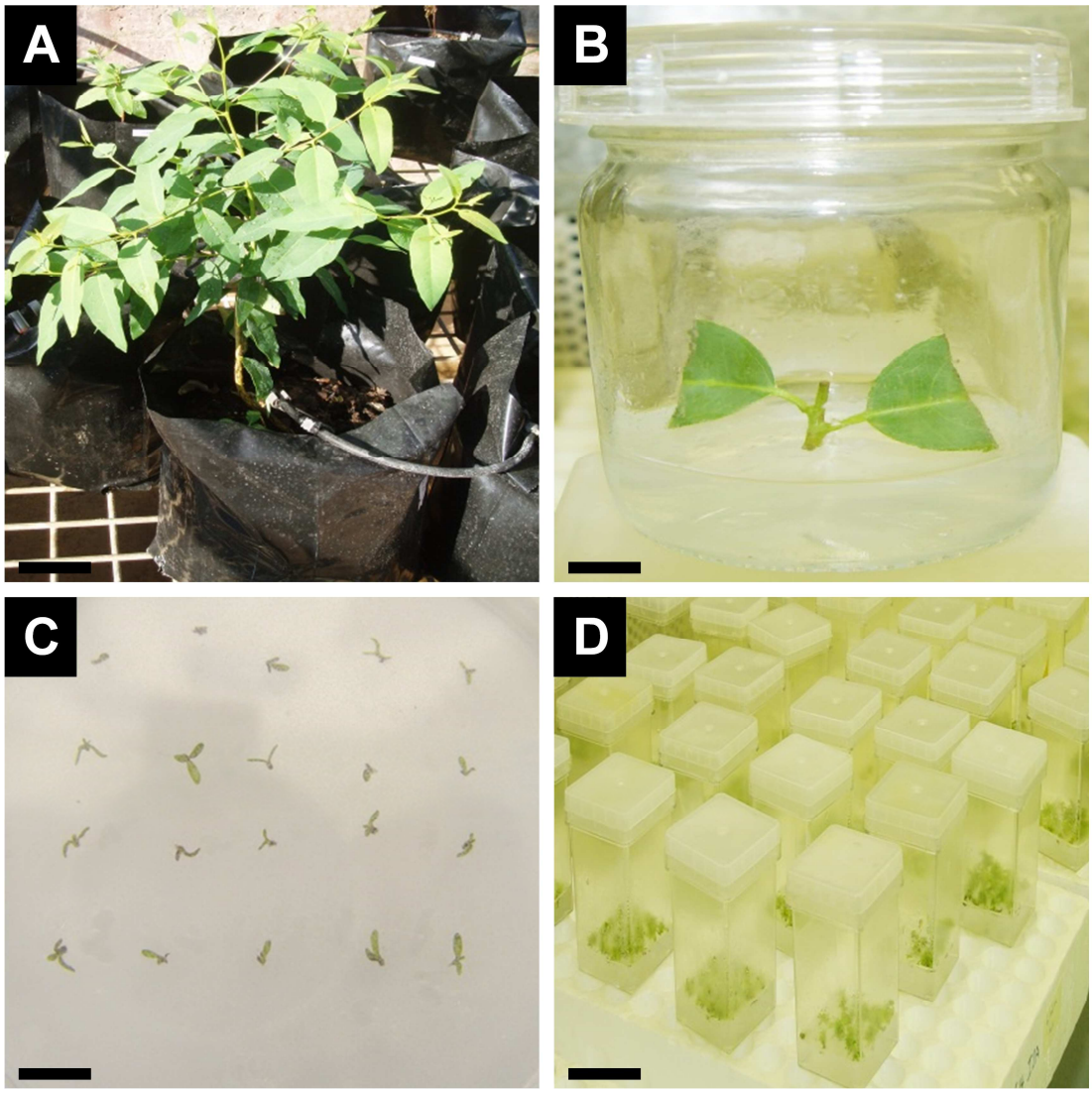


Figure 2.1. Establishment of *E. grandis in vitro* plant material. (A) *E. grandis* greenhouse parent plant. (B) Surface sterilized micro-cuttings obtained from parent plants. (C) Buds from sterile micro-cuttings were multiplied *in vitro*. (D) Shoot clusters from *in vitro* material were subcultured regularly. Scale bar: 1cm represents 4cm (A), 1.5cm (B), 0.7cm (C) and 10cm (D).

An established protocol was used to generate *in vitro* cultures throughout the duration of the project (Watt *et al.*, 1995). Shoots were obtained from the parent plants (Figure 2.1A) and transferred to a laminar flow bench for the remainder of preparative treatments. After trimming the material was surface-decontaminated in 0.02% (w/v) mercuric chloride and 10 $g.l^{-1}$calcium hypochloride for 10 minutes each, with thorough rinses in between and after in sterile ultrapure water (MilliQ®). The surface decontaminated shoots were trimmed down to the nodal areas and cultured for 7 days on 20ml of semi-solid multiplication medium (Murashige and Skoog [1962] salts and vitamins supplemented with 25$g.l^{-1}$ sucrose, 3$g.l^{-1}$ Gelrite®, 0.35$mg.l^{-1}$ benzylamino purine [BAP], 0.01$mg.l^{-1}$ naphthylacetic acid [NAA], 0.1$mg.l^{-1}$ biotin and 0.1$mg.l^{-1}$ calcium pentothenate, pH 5.6-5.8) in 100ml glass culture bottles (Figure 2.1B) to promote formation of buds (*Watt et al*., 1995). Buds were excised and cultured on fresh multiplication medium (Figure 2.1C) to induce shoot-cluster formation. Shoot clusters (Figure 2.1D) were established within eight to ten weeks. All *in vitro* cultures were maintained in the growth room at 25ºC ±2ºC, with a photon flux density of 100$\mu mol.m^{-2}.s^{-1}$, under 14h light and 10h dark photoperiod. Cultures were routinely subcultured onto fresh medium every eight weeks and maintained under the standard growth room conditions described above.

Shoot clusters were treated only when necessary with antibiotic solution consisting of 200ml MS salts with vitamins, filter-sterilised 5$mg.l^{-1}$ Gentamycin, 4$mg.l^{-1}$ Rifampicin and pH adjusted to 4.3. Shoot clusters were transferred into a conical flask with sterile forceps, covered with foil and placed on a Labcon® orbital shaker (75rpm) for 15. Shoots were then removed from the solution with sterile forceps, blotted dry on sterile paper and plated onto multiplication medium.

2.2.2 Pretreatment of *in vitro* material with abscisic acid

Shoot-clusters containing multiple axillary buds were transferred onto semi-solid hormone-free Murashige and Skoog, 1962 (MS) medium with vitamins supplemented with 25$g.l^{-1}$ sucrose and 3$g.l^{-1}$ Gelrite® (acclimation medium) in Magenta jars for 7 days, and then transferred onto the same medium supplemented with 5$mg.l^{-1}$ ABA for 5 days. Axillary buds isolated from clusters grown for 5 days on acclimation medium were used as ABA-untreated controls.

2.2.3 Water content assessment, vigour and viability of *in vitro* material

Buds (1-2mm) were excised from the shoot clusters under sterile conditions and used as the experimental samples. The average water concentration of both ABA pre-treated and untreated buds was determined gravimetrically. Individual buds were dried in an oven at 80ºC for 24h and the water concentration calculated as $g.g^{-1}$. The water content was expressed on a fresh mass basis (FMB) because this method is conventional in cryopreservation. Viabilty was determined before and after drying by plating for regrowth onto 20ml hormone-free acclimation medium in Petri dishes (90mm in diameter). Viability was recorded 4 weeks after plating by visual assessment to determine the survival percentage. Vigour was also determined, by assessing the number of buds produced per experimental bud.

2.2.4 Drying and re-imbibition of *in vitro* material

Axillary buds were isolated from both acclimated shoot clusters (control) and shoot clusters maintained on ABA-enriched medium for 5 days. Buds were placed on sterile foil open boats (2 cm) and partially dried over 300g of activated silica gel in an airtight 4500cm^3 desiccator for 20min. The silica gel was acclimated to 25ºC, 24 h prior to the drying procedure. Dried buds were then rehydrated by plating on acclimation medium.

2.2.5 Microscopy

The microscopy studies focused on the meristematic cells of axillary buds. The following treatments were investigated:

- hydrated untreated control (light and transmission electron microscopy)
- hydrated ABA pre-treated (light and transmission electron microscopy)
- partially dried untreated control (20min over silica gel)
- partially dried ABA pre-treated (20min over silica gel)
- 24h rehydrated untreated control (light and transmission electron microscopy)
- 24h rehydrated ABA pre-treated (light and transmission electron microscopy)
- 24 – 96h rehydrated untreated control (fluorescence microscopy, performed episodically)
- 24 – 96h rehydrated ABA pre-treated (fluorescence microscopy performed episodically at every 24 hours)

- Hydrated untreated control [fluorescence microscopy, 4,6-diamidino-2-phenylindole (DAPI)]
- Hydrated ABA pre-treated [fluorescence microscopy (DAPI)]
- 24h rehydrated untreated control [fluorescence microscopy (DAPI)]
- 24h rehydrated ABA pre-treated [fluorescence microscopy (DAPI)]

2.2.5.1 Light and transmission electron microscopy

The ultrastuctural studies were used to assess the response of both ABA pre-treated and untreated (control) excised buds to drying. Axillary buds were prepared for microscopy using a standard gluteraldehyde-osmium method (Appendix) and embedded in Spurr's epoxy resin (Spurr, 1969). Semi-thin (0.5µm – 0.8 µm) and ultra-thin (60nm-70nm) sections of the axillary buds were obtained using a Reichert Ultramicrotome. Semi-thin sections were heat-fixed onto 76 x 26mm glass slides and stained with 0.01% (w/v) Toluidine Blue. These were then mounted in *p*-xylene-*bis*(*N*-pyridinium bromide) (DPX) and covered with glass cover slips, and thereafter viewed and photographed with an Olympus BH-2 compound microscope with an attached Nikon DXM1200 digital camera. Ultra-thin sections were collected on 200µm mesh copper grids and stained with lead citrate [Appendix (Reynolds, 1963)]. These sections were viewed and photographed with a JEOL 100-S transmission electron microscope with the focus emphasis on the meristem cells. The observation on partially dried buds was done with caution because of the possible rehydration artefacts that may have occurred during preparation.

2.2.5.2 Fluorescence microscopy

The vital stains, 4,6-diamidino-2-phehylindole (DAPI) and propidium iodide (PI), were used to identify nonviable and viable cells within axillary buds. DAPI (a blue fluorescent probe that stains the nuclei) detected living cells, whereas PI (a red fluorescent probe that also stains the nuclei) detected dead cells (Suzuki *et al*., 1997; Zhang *et al*., 2005). Investigations using both stains were performed after 24h rehydration, and with the PI stain episodically over 24h to 96h of rehydration on acclimation medium. Stains were prepared in 10Mm Tris-$MgCl_2$, pH 7.5 buffer and used at $1\mu l.ml^{-1}$. Excised buds were trimmed with a surgical blade to expose the leaf primordia and meristem. These were then incubated in the vital stains for 15 min in

DAPI and 5 min in PI, in the dark at 25ºC in Petri dishes (35mm in diameter). Thereafter, PI stained buds were mounted in glycerol on a glass bottom uncoated microwell dish (35mm in diameter), then viewed and imaged with a Zeiss LSM-410 laser scanning confocal microscope (excitation/emission 536/617nm). Overlay projection images were produced. The DAPI stained buds were mounted in glycerol on a glass slide and covered with a cover slip, then viewed and imaged with a Zeiss Axiophot stereomicroscope with a blue filter set 09 (BP~ 450-490, FT~ 510 and LP~ 515). Transmission images were also produced. All images (DAPI and PI) were taken within 15min of staining.

2.2.6 Data and statistical analyses

Representatives of histological and ultrastructural data are presented in micrographs, and each experiment was replicated three times (n=20 buds per experiment). Water content, viability and vigour tests were replicated three times and each replicate included 20 axillary buds. Average values and standard errors were calculated for the survival percentages on all regrowth experiments.

2.3 RESULTS AND DISCUSSION

2.3.1 Water content analyses and drying of axillary buds

Eucalyptus grandis is described as a disease-intolerant species and sensitive to dry conditions (Dvorak, 2012). *In vitro* material such as axillary buds is prone to rapid wilting outside the *in vitro* environment due to the poor development of structures such as functional cuticles to minimize water loss (Padayachee *et al*., 2008, 2009). The inability of such *in vitro* material to withstand the extensive drying required for cryopreservation maybe overcome by the exogenous application of ABA prior to cryopreparative drying (Cruz-Cruz *et al*., 2013).

The *E. grandis* untreated and ABA pre-treated buds that were isolated from the *in vitro* shoot clusters were found to be highly hydrated, with average water contents of $0.76g.g^{-1}$±$0.02g.g^{-1}$ (FMB). The partial drying procedure for *E. grandis* axillary buds involved the exposure of buds to activated silica gel in an airtight desiccator for 20min. This rapid drying method was found to be suitable for drying *in vitro* axillary buds as it resulted in the maintenance of high viability at low water concentration when compared with equilibrium drying over saturated salt solutions creating specific relative humidities (slow drying achieved over days) (Mlambo, 2004; Padayachee, 2007). Similarly, rapid drying has been shown to be suitable for the cryo-preparation of embryonic axes isolated from recalcitrant seeds (Berjak *et al*., 1990; Berjak *et al*., 1999) and cassava somatic embryos (Stewart *et al.*, 2001). During the process of drying, the silica gel absorbed the water from the tissues. This has been associated with an increase in cytoplasmic viscosity and the promotion of the transition of water to the glassy state (Proctor *et al*., 2007; Wood, 2007; Yamazaki *et al*., 2009). This was reflected as a decrease in water content from an average of $0.76g.g^{-1}$ ± $0.02g.g^{-1}$ in both ABA pre-treated and untreated buds to $0.45g.g^{-1}$ ± $0.02g.g^{-1}$(ABA pre-treated) and $0.33g.g^{-1}$ ± $0.01g.g^{-1}$ (untreated). These results showed that the application of $5mg.l^{-1}$ ABA for 5 days induced some resistance to water loss whilst better retaining viability (Figure 2.2), 70% ± 0.04% (ABA pre-treated) compared with 55% ± 0.04% (untreated). The vigour of the surviving ABA pre-treated buds was not affected as compared with the untreated control; in 4 weeks the rehydrated untreated buds produced an average of 2.5 buds ± 0.5 buds, whereas the ABA pre-treated buds produced 3.5 buds ± 0.2 buds ($p<0.05$).

Exogenous application of ABA is known to increase stress tolerance by triggering the production and accumulation of various biomolecules, including protective proteins such as Late Embryogenesis Abundant (LEA) proteins, LEA-like proteins and other compatible solutes which collectively increase plant stress tolerance (Tunnacliffe and Wise, 2007; Shih *et al.*, 2010). Consequently, it has been used extensively in *in vitro* culture to induce tolerance to desiccation (e.g. Stewart *et al*., 2001; Sreenivasulu *et al*., 2007; Zou *et al.*, 2009). Late Embryogenesis Abundant proteins are glycine rich, highly hydrophilic and remain soluble even when boiled (Shih *et al*., 2010). Furthermore, they have randomly-coiled regions that are suggested to function in binding water and thus, maintaining the minimum cellular water requirements (Ingram and Bartels, 1996; Shih *et al*., 2008). The random coils of LEA-like proteins permit their shape to conform to that of other solutes and, therefore provide a cohesive layer which provides stability in the cells (Shih *et al*., 2008, 2010). In this study, the partial tolerance to water loss and maintenance of vigour suggested that the ABA pre-treatment activated ABA-dependant cellular protective mechanisms to avoid damage, although such mechanisms were not determined in this study. Nevertheless, the observed increased viability of axillary buds treated with ABA suggested that *E. grandis* buds had the ability to activate one or more ABA-dependent cellular protective mechanisms which promoted some resistance to water loss.

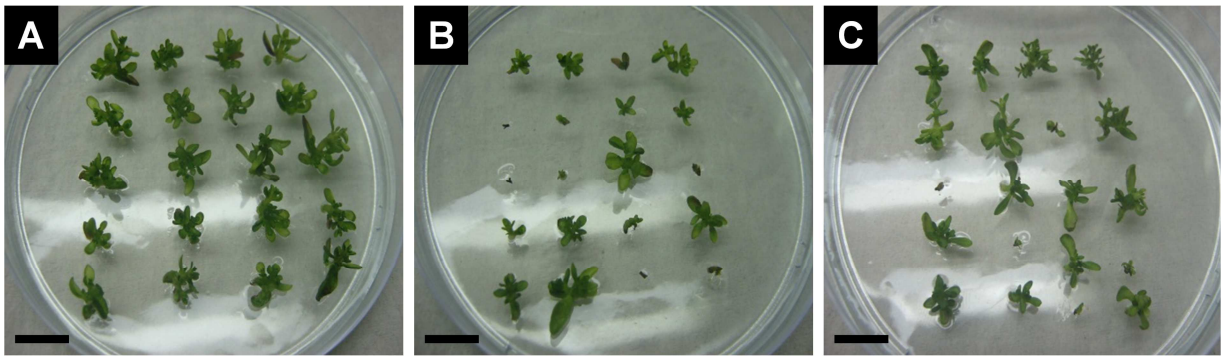


Figure 2.2. *Eucalyptus grandis* hydrated (A) and rehydrated (B-C) shoot clusters. *In vitro* axillary buds were partially dried over activated silica gel for 20 minutes and rehydrated on growth medium for 4 weeks. (A) Shoots from ABA untreated hydrated buds with an average water content of 0.76g.g^{-1} ± 0.02g.g^{-1} (FMB) and 100% viability. (B) ABA Untreated buds that were partially dried down to 0.33 g.g^{-1} ± 0.014g.g^{-1} and rehydrated, retained 55%± 0.04% viability. (C) ABA Treated buds that were partially dried down to 0.45g.g^{-1} ± 0.02g.g^{-1} retained a higher viability of 70% ± 0.04%. These results showed that the application of 5mg.l^{-1} ABA for 5 days induced some resistance to water loss whilst better retaining viability, compared with the untreated (rehydrated) control. Twenty excised buds were used per experiment (n=20). Each experiment with untreated and ABA pre-treated buds was repeated three times. Scale bar: 1cm represents 1.8cm.

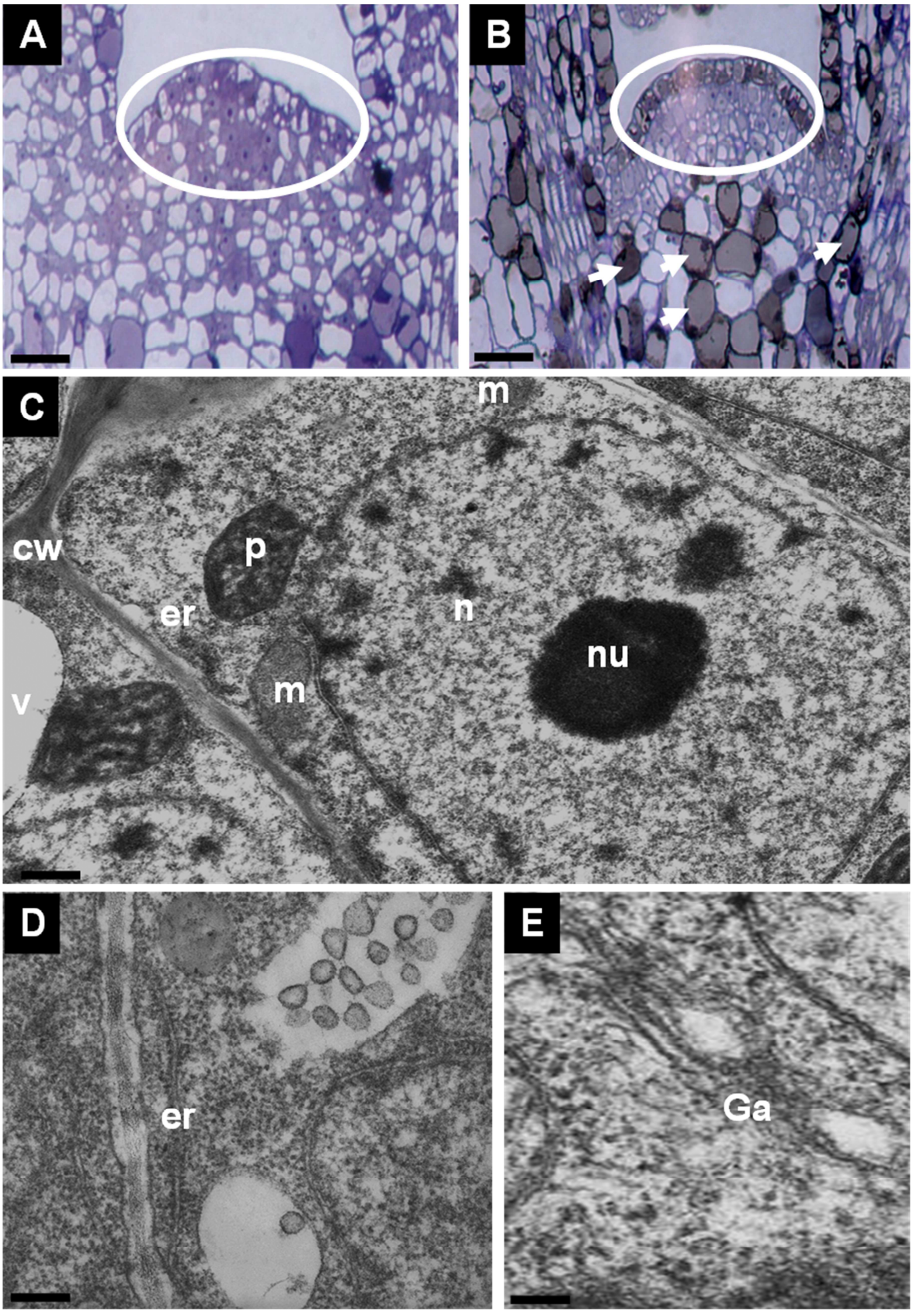
In this regard, phenolic compounds are secondary metabolites that have diverse biological activities such as being part of the defence mechanisms and antioxidant systems (Peinado *et al.*, 2009; Delazar *et al.*, 2010; Valentão *et al.*, 2010). Phenolic accumulation is well characterised in tissues of plants under stress, and is accepted as an indication of the triggering of stress response pathways (Gechev *et al*., 2012). Phenolic compounds (e.g. flavonoids) are known to have antioxidant properties and are usually produced in response to the oxidative burst reported to occur as part of the stress signalling pathway (Peinado *et al*., 2009; Gechev *et al*., 2012). Therefore, the apparent accumulation of phenolic compounds in the ABA pre-treated hydrated buds (Figure 2.3B) provided further evidence, albeit only visual, of the activation of such protective mechanisms.

2.3.2 The effect of ABA on the responses of *E. grandis in vitro* axillary buds to drying and rehydration

A histological survey of 60 buds showed a similar appearance between the meristems and associated tissues of both the ABA pre-treated buds and those of the control (figure 2.3A and B). The axillary bud meristem is composed of distinct layers of cells, i.e. the epidermal layer, the subepidermal tunica and the corpus (Beck, 2010). In *E. grandis* axillary buds, the tunica consisted of 1 or 2 layers, subtended by 3 to 4 layers of the corpus (encircled areas on Figure 2.3A and B). The meristematic dome (tunica and corpus) of the fully hydrated ABA pre-treated and untreated buds consisted of cells that were typically meristematic with large nuclei, small vacuoles, mitochondria with dense matrices and distinct cristae, and endoplasmic reticulum which generally occurred close to the plasma membrane (Figure 2.3C-E). The meristematic cells contained nucleoli which were well defined and comparatively large (Figure 2.3C), compared with more mature cells where these structures are less evident. Active protein production in cells requires the presence of nucleoli, ribosomes, polysomes, endoplasmic reticulum and Golgi apparatus; the observation of these features in *E. grandis* hydrated cells was therefore evidence that proteins were synthesised and processed to their functional sites (figure 2.3C- E).

The energy required for protein synthesis and other cell metabolic activities is produced in part, by the mitochondria (Taylor *et al*., 2004; Wang *et al*., 2012; Youle and van der Bliek, 2012).The observed mitochondria in *E. grandis* were characterised by outer membranes and invaginated, well-defined cristae (Figure 2.3C). Collectively, the histological and ultrastructural observations of *E. grandis* meristematic cells (Figure 2.3) indicated active metabolism.

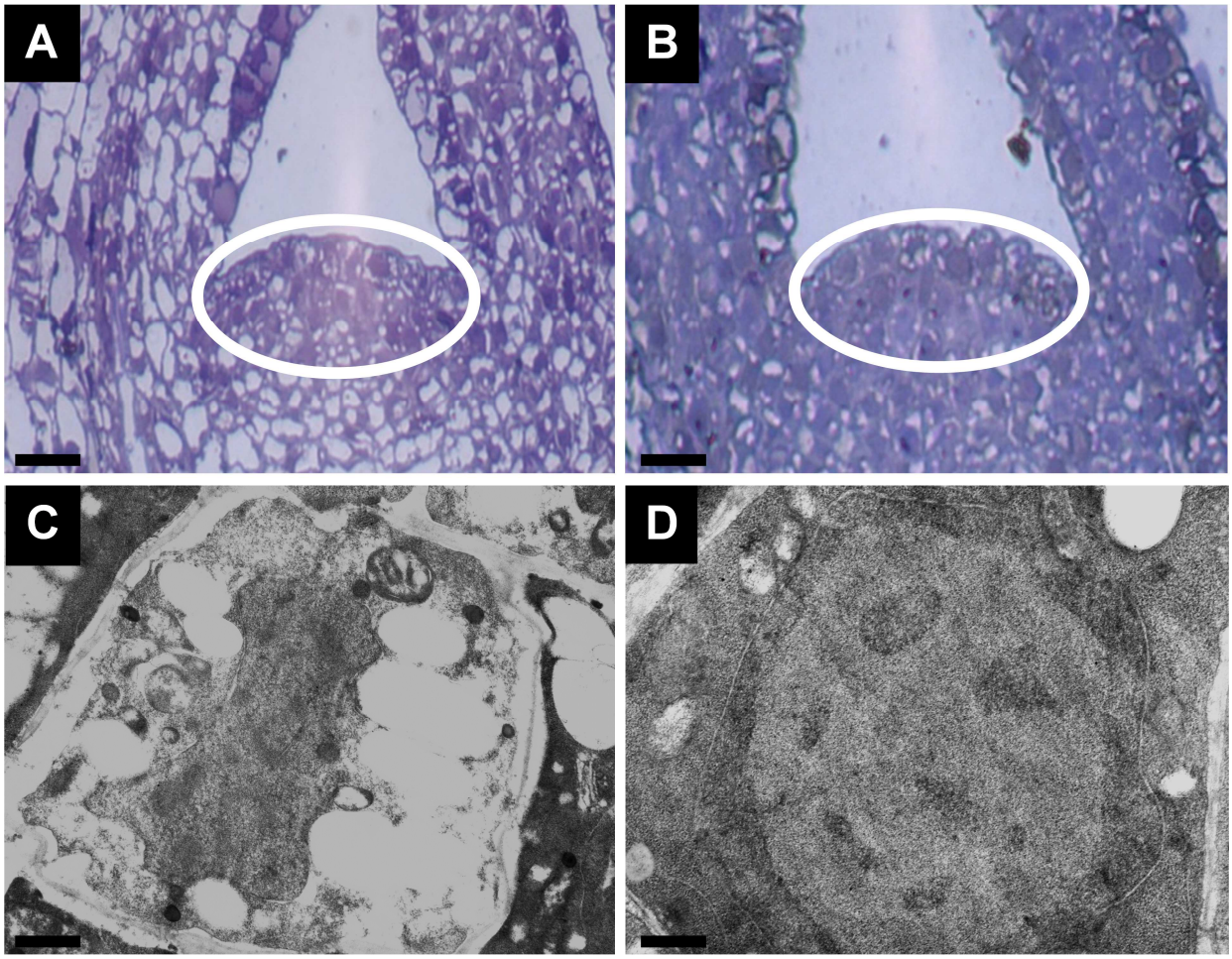
Figure 2.3. Micrographs of hydrated untreated and ABA pre-treated *E. grandis in vitro* axillary buds with an average water content of 0.76g.g^{-1} ± 0.02g.g^{-1} (FMB) and 100% viability. Light micrographs of untreated (A) and ABA pre-treated (B) buds showing the leaf primodia and meristematic cells (encircled areas). The accumulation of phenolics in the epidermal layer of the meristematic dome and other areas of the bud (examples are highlighted by arrows) was observed in the ABA pre-treated buds (B); no other visible differences were noted between the hydrated ABA pre-treated (B) and untreated (A) buds. Ultrastructural investigations focussed on the meristem region. Meristematic cells in both hydrated untreated and ABA treated buds showed similar appearance and were typically meristematic (C - E) with large, well-defined nuclei (n) and nucleoli (nu), small vacuoles (v), mitochondria (m) with dense matrices, endoplasmic reticulum (er) which generally occurred close to the plasma membrane, proplastids (p) distinct cell walls (cw) and Golgi apparatus (Ga). Twenty excised buds were used per experiment (n=20). Each experiment on untreated and ABA pre-treated buds was repeated three times. Scale bar: 1cm 25µm (A-B), 1.5µm (C), 0.48µm (D) and 0.14µm (E).


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In contrast, the dried untreated buds showed extensive damage or complete loss of cellular integrity (Figure 2.4A and C), whereas, ABA pre-treated buds appeared to have moderately damaged cells (Figure 2.4 B and C). Cells in untreated buds exhibiting extensive drying damage were characterised by very little subcellular structure and severe plasma separation (Figure 2.4C). The moderately damaged cells in the treated buds, however, displayed visible nuclear profiles and damaged mitochondria, indicated by the loss if internal structures (Figure 4D). The observation of the damage in both dried (20min) treated and untreated buds was noted with caution due to the possible rehydration artefacts that may have occurred during the preparation of such tissues for microscopy (Wesley-Smith, 2001). Dried plant material that has been prepared for TEM using aqueous-based preparative methods, may result in uncertainty concerning the extent of rehydration that occurs during tissue fixation and subsequent processing. Rehydration of dried plant cells may occur within seconds or minutes following immersion into aqueous medium (Thomson and Platt, 1997; Wesley-Smith, 2001). For that reason, aqueous-based fixatives are unlikely to provide an accurate impression of cells as they occur in the dried state (Platt *et al*., 1997; Wesley-Smith, 2001). The use of cryofixation and freeze substitution is believed to better identify the stage of damage in dried tissues (Takeuchi *et al*., 2010). However, as such technology was not available for the present study, undertaken investigations into the effect and responses to rehydration. That is, the material was allowed to imbibe in culture before being fixed and processed for microscopy.

The rehydrated bud tissues also showed a variation in the proportion of intact cells compared with damaged cells (Figure 2.5). The rehydrated untreated buds, that represented the 45% ± 0.04% nonviable material, showed lethally damaged meristematic cells (Figure 2.5A). These were characterized by disintegrated nuclei, plasma membrane separation and withdrawal from the cell wall, very little subcellular structure, and in extreme cases total loss of cellular integrity (Figure 2.5A and C). The cell walls, however, appeared intact, indicating a high degree of rigidity. Although the outline of the nuclear profile was visible in some of these cells, disorganization of the cell organelles and loss of cell integrity were some of the reasons for the loss of viability. Such extreme damage was reported in some cells that are typically intolerant to dry conditions (Atkin and Macherel, 2009).

Figure 2.4. *Eucalyptus grandis in vitro* axillary buds partially dried for 20min over activated silica gel. Untreated dried buds (A) appeared to be more damaged than the ABA pre-treated buds (B). The meristem cells of untreated dried buds (C) showed extensive and/or complete loss of cellular integrity, whereas, the ABA pre-treated dried buds (D) showed moderately damaged cells. The damage observed in the partially dried buds was noted with caution because of the possible rehydration artefacts that may have occurred during the preparation of buds for microscopy. Twenty excised buds were used per experiment (n=20). Each experiment on untreated and ABA pre-treated buds was repeated three times. Scale bar: 1cm represents 25µm (A-B), 4.4µm (C) and 3.2µm (D).


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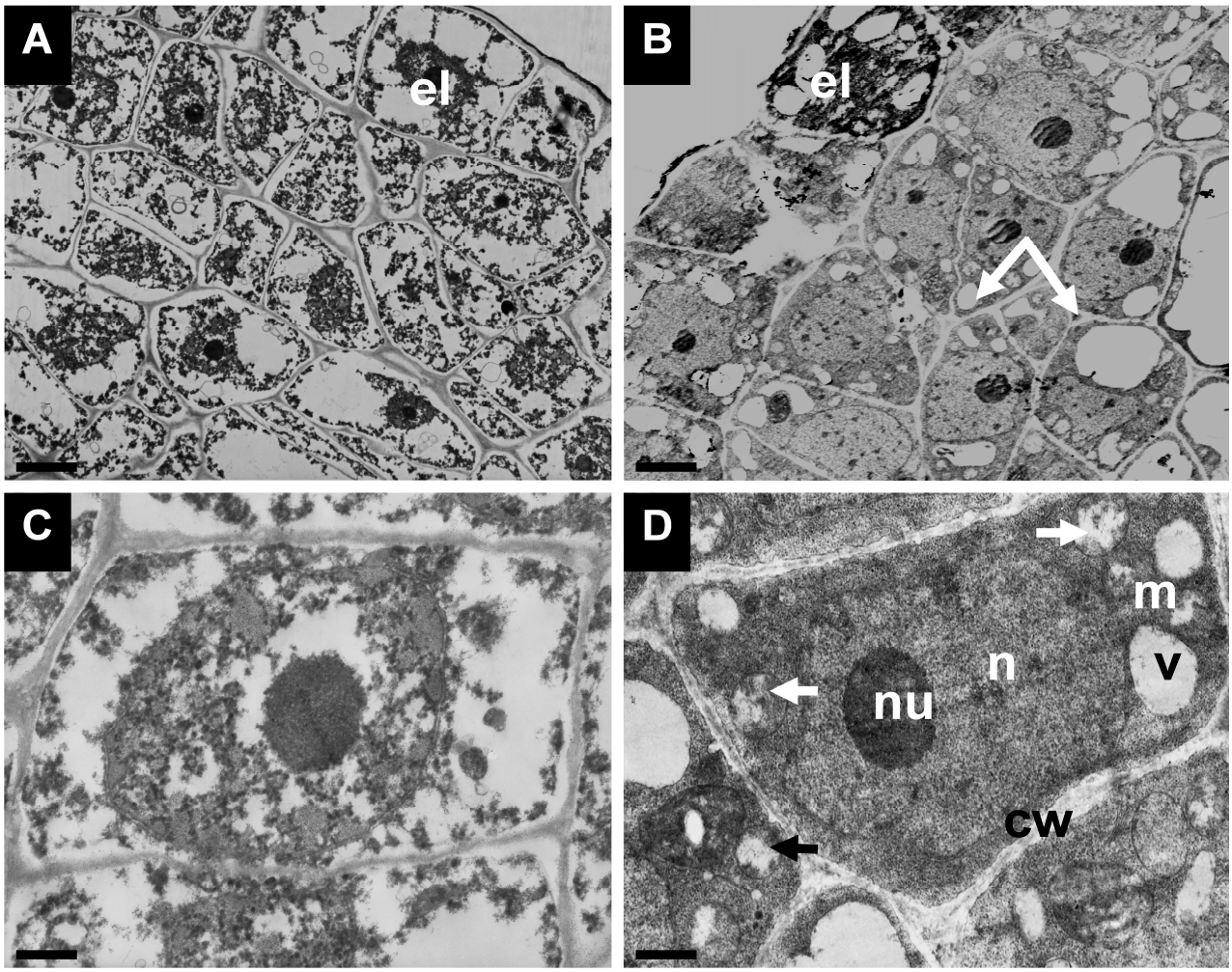
Tolerance to drying involves the ability for preservation of cell integrity during drying, and if damage occurs, the repair mechanisms should be available to restore cellular integrity (Berjak *et al*., 1984). The cytoskeleton is important in maintaining cell integrity (Lee *et al*., 2007). Nuclear lamins associated with the nuclear envelope, are related to the intermediate filaments that form part of the cytoskeleton (Wightman *et al*., 2009; Beck, 2010). They also provide skeletal support that reinforces the nuclear envelope and the linkages holding the nucleus in position in the cytoplasmic filament network. Furthermore, lamins serve as linkers between the nuclear envelope and chromatin fibres, thus maintaining parts of the chromosomes in the nucleus (Berjak *et al*., 1984; Gunning and Steer, 1996; Wightman *et al*., 2009; Beck, 2010). Disruption of the cytoskeleton and associated lamins would therefore result in deranged cellular activity. In this regard, the collected data suggested that the drying process possibly had an effect by disrupting this complex cellular component (Mycock *et al.,* 2000; Beck, 2010). It is also clearly demonstrated that the sensitivity of *E. grandis* axillary buds to drying is due to tissues lacking effective mechanisms to retain integrity during drying or mechanisms to restore integrity during rehydration.

The meristems of the ABA pre-treated nonviable buds (30% ± 1.55%) exhibited a mosaic pattern in cellular integrity after 24h rehydration *in vitro* (Figure 2.5B). The extent of the damage ranged from intact cells with minimal injury to severely damaged cells (Figure 2.5B). In damaged cells, the effect of drying was more extreme upon rehydration being characterised by chromatin clumping, plasmolysis and very little subcellular structure. Although the outline of the nuclear profile was visible, cells displayed loss of cellular integrity upon rehydration.

Intact cells in ABA pre-treated buds were similar to hydrated cells, however organelles exhibited some of the symptoms of drying stress (Figure 2.5D). The mitochondria for example, were characterised by cleared matrices and disrupted cristae (Figure 2.5D) (highlighted by arrows). However, the extent of the inner membrane clearing varied in intact cells. The loss of the inner membrane definition in the mitochondria indicated that the mitochondrial status in *E. grandis* was an effective indicator of the drying stress. This has been shown in other tissues such as those of somatic embryos of *Manihot esculenta* (Stewart *et al.*, 2001) and *Avicennia marina* (Berjak *et al.*, 1984). The inner membranes of the mitochondria (cristae) contain enzymes and prosthetic groups that are involved in the electron transport

system, along with ATPase activity. These enzymes are tightly bound or integral to the cristae and remain active in the electron transport and phosphorylation only as long as the inner membrane is intact (Taylor *et al*., 2004). Damage to the mitochondrial membranes results in the release of the enzymes and substrates of the citric acid cycle from the mitochondrial matrix (Navrot *et al.*, 2007). Concomitantly, the loss of the proton gradient across the inner mitochondrial membrane reduces or even halts ATP production (Zsigmond *et al.*, 2008). More importantly, it can result in oxidative stress leading to reactive oxygen species (ROS) accumulation, which can include hydrogen peroxide, hydroxyl radicals and super anions (Roach *et al.*, 2010). Cellular membranes are a target for ROS, and tissues that are sensitive to drying can lose integrity as a result of activities of ROS during drying (Taylor *et al*., 2004; Roy *et al*., 2008; Blokhin and Fagerstedt, 2010; Mittler *et al*., 2011).

Figure 2.5. Subcellular features of *E. grandis* meristem cells in rehydrated (24h) untreated and ABA pre-treated buds. Meristem cells (A and C) of partially dried (down to 0.33 $g.g^{-1}$ ± 0.014$g.g^{-1}$) and rehydrated (24h) untreated buds displayed loss of cellular integrity; however, the outline of the nuclear profile was visible in some of the cells (C). The buds were presumed to be representative of the 45% ± 0.04% nonviable material because of the extensive subcellular disruption. In ABA pre-treated nonviable buds (30% ± 1.55%), a mosaic of intact and damaged meristem cells were observed (B) (indicated by the arrows). Intact cells (D) displayed well-defined organelles, whereas damaged cells showed complete loss of cellular integrity (D) (indicated by the arrow). Increased degree of clearing of the mitochondrial matrices in the intact cells was considered as an indication of dehydration stress (D, highlighted by arrows). These ABA pre-treated buds were presumed to be representative of the 30% ± 1.55% nonviable material. Abbreviations: el, epidermal layer; cw, cell wall; m, mitochondrion; n, nucleus; nu, nucleolus; nm, nuclear membrane. Twenty excised buds were used per experiment (n=20), and each experiment on untreated and ABA pre-treated buds was repeated three times. Scale bar: 1cm represents 14.5µm (A-B) and 1.5µm (C-D).


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The use of the vital stains, DAPI and PI, in both ABA pre-treated and untreated (rehydrated) buds confirmed and supplemented the results that were obtained by the light and transmission electron microscopy studies. The DAPI stain showed only intact meristematic cells of the ABA pre-treated buds that were rehydrated for 24 hours. (Figure 2.6D) (Figure 2.6C is a transmitted light image of figure 2.6D). Cell viability was demonstrated by bright-blue fluorescence in the intact cells. However, fluorescence was not detected in 45% ± 0.04% of the rehydrated (24h) untreated buds (Figure 2.6B) (Figure. 2.6A is a transmitted light image of figure. 2.6B), which attested to the loss of viability of the majority of untreated buds after drying (100 to 55% ± 0.04%). These buds were presumed to be representative of the 45% ± 0.04% untreated rehydrated buds. The fluorescence that was detected in the intact meristematic tissues of the rehydrated ABA pre-treated buds did not only confirm the mosaic pattern that was observed in the ultrastructure of the meristems of the rehydrated ABA pre-treated buds, but also showed the differences in the responses to the drying of the component tissues of the buds.

When using the PI stain, nonviable tissues in both ABA pre-treated (Figure 2.6H) and untreated (Figure 2.6F) rehydrated whole buds were shown by a bright-red fluorescence (Figure 2.6E and G are overlay projections of figure 2.6F and H, respectively). Therefore, these buds were also presumed to be representative of the 45% ± 0.04% untreated and 30% ± 1.55% ABA pre-treated rehydrated buds. In the ABA pre-treated buds, the stain not only validated the survival of some meristematic cells (encircled areas in Figure 2.6H), it also showed that the leaf primodia of the buds (treated and untreated) were lethally impacted by drying (Figure 2.6F and H). This rapid and lethal response was indicative of necrosis (Reape *et al.*, 2008). It was also noted that although some of the meristematic domes in the ABA pre-treated buds retained viability for up to 72h of rehydration (Figure 2.7B and D) (Figure 2.7A and C are overlay projections of figure 2.7B and D, respectively), they eventually died after 96 hours (Figure 2.7F) (Figure 2.7E is an overlay projection of Figure 2.7F). The red fluorescence was not detected in those meristematic cells after 24, 48 and 72h of rehydration; thereby indicating viability of those meristematic cells at those rehydration stages (Figures 2.6H, 2.7B and D). This suggested that ABA application delayed the death of those cells for up to 72h (Rai *et al.*, 2011).

Mitochondria are important sources of ROS, which are thought to act as signals during plant PCD regulation (Navrot *et al.*, 2007; Zsigmond *et al.*, 2008; Roy *et al.*, 2008, Doyle *et al.*, 2010; He *et al.*, 2012). An increased production of ROS due to biotic or abiotic stresses may result in a release of cytochrome *c* from the mitochondria into the cytosol and a subsequent activation of caspase-like proteases, which have been shown to be involved in the control of death activation in plants (Roy *et al.*, 2008; Roach *et al.*, 2010; Serrano *et al.*, 2012). The observed changes in the mitochondrial profiles of intact cells of the meristematic domes of dried axillary buds (Figure 2.5D) could indicate that the drying process resulted in stresses and/or disruptions of the ATP generation processes which lead to more production of ROS and subsequently activated the death process.

Figure 2.6. Micrographs of *E. grandis in vitro* untreated and ABA pre-treated axillary buds stained with DAPI (A-D) and PI (E-H) after rehydration for 24 hours. A and C are transmitted light images of B and D, respectively. The DAPI stain enters into living cells and reacts with nucleic acids. This was demonstrated by a bright-blue fluorescence of the nuclei. Fluorescence was not detected in the nonviable untreated buds (B). This confirmed the loss of viability of the 45% ± 0.04% of untreated buds. In contrast, fluorescence was detected in the intact meristematic tissues of nonviable (30% ± 1.55%) ABA pre-treated buds (D) [encircled area on the transmission image (C)]. This confirmed the mosaic pattern that was observed in the ultrastructure of the meristems of the ABA pre-treated buds. PI enters into dead cells, reacts with nucleic acids and shows red fluorescence. E and G are overlay projection images of F and H, respectively. Nonviable tissues in both untreated (F) and ABA pre-treated (H) rehydrated whole buds were shown by a bright red fluorescence. The inability of the PI stain to enter some meristematic cells confirmed the survival of those cells in ABA pre-treated buds (H) (encircled area). The stain also showed that all the areas in untreated buds (F) and the leaf primordia of treated buds (H) were more prone to lethal drying damage. Therefore, these buds were presumed to be representatives of the 45% ± 0.04% (untreated) and 30% ± 1.55% (ABA pre-treated) nonviable material dried for 20min over silica gel and rehydrated for 24h. Twenty excised buds were used per (DAPI and PI stain) experiment (n=20), and each experiment on untreated and ABA pre-treated buds was repeated three times. Scale bar: 1cm represents 10µm and 5.5µm (D).

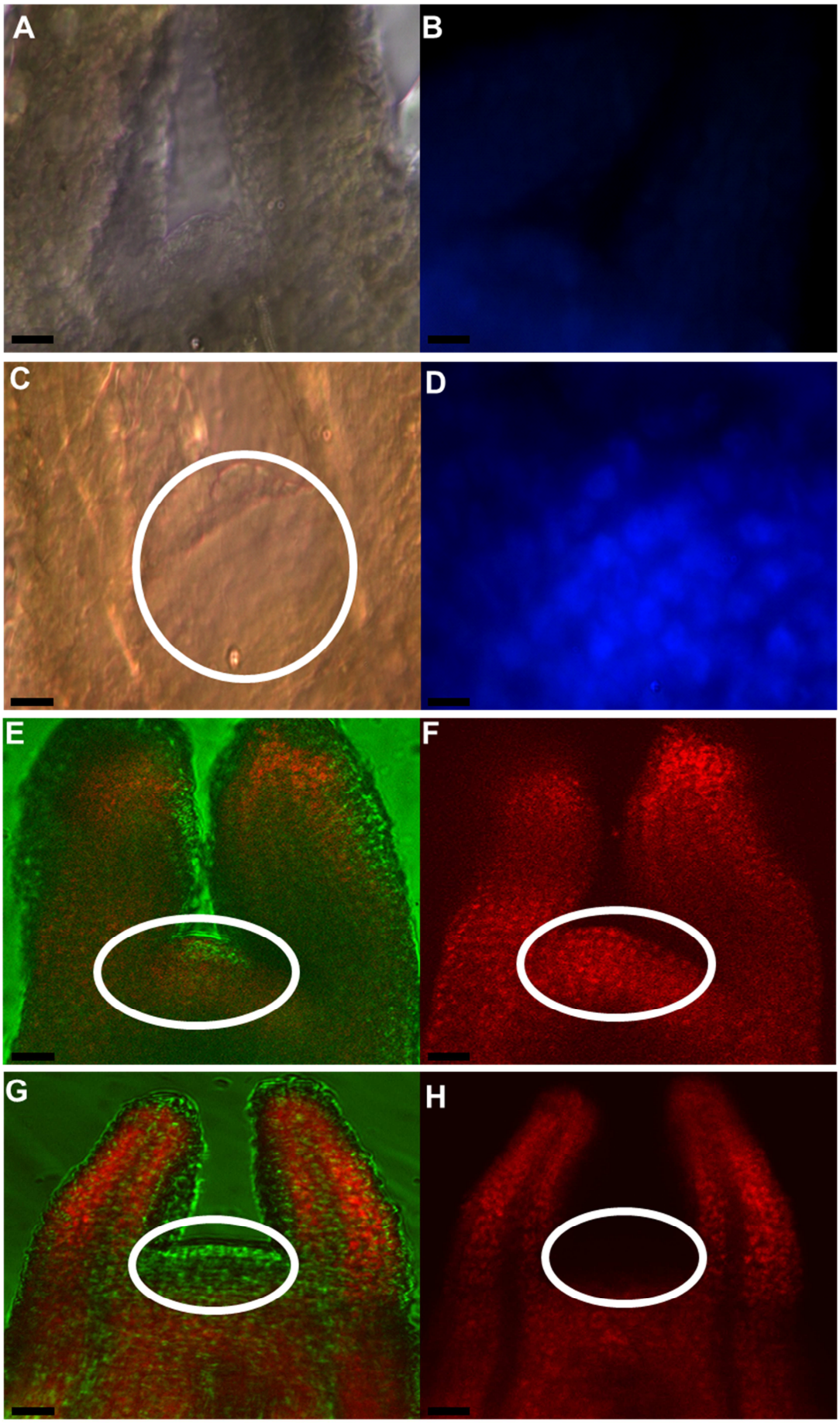
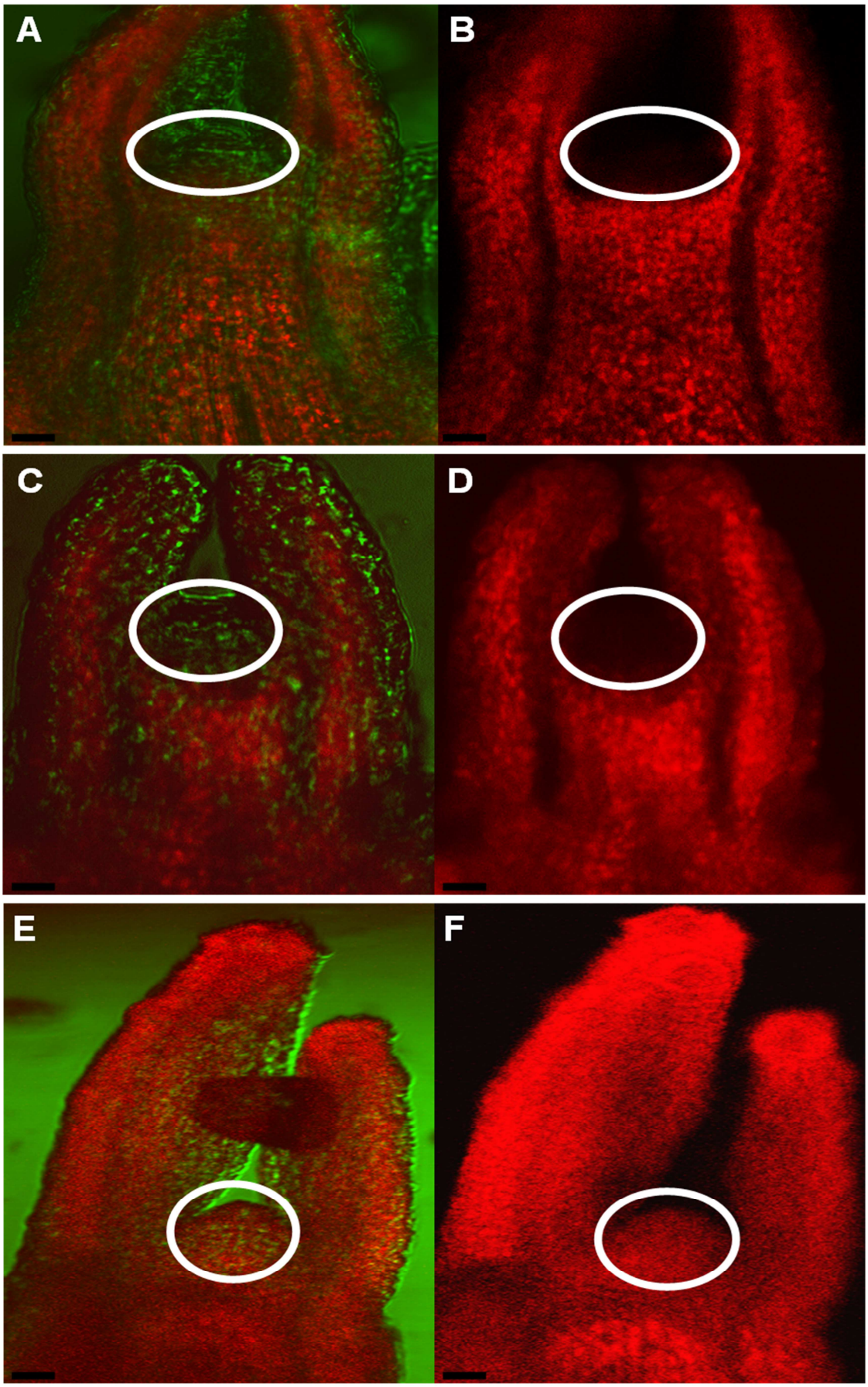

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Figure 2.7. Micrographs of *E. grandis in vitro* ABA pre-treated nonviable axillary buds stained with PI (enters into dead cells, reacts with nucleic acids and shows red fluorescence) after 48 h (B), 72h (D) and 96h (F) rehydration. A, C and E are overlay projection images of B, D and F, respectively. These buds were presumed to be representatives of the 30% ± 1.55% ABA pre-treated nonviable rehydrated buds. Intact meristematic cells were able to maintain viability for up to 72h of rehydration (B and D). This was indicated by the inability of the stain to enter those cells during that period (encircled area). However, such cells eventually died after 96h (F) (encircled area). This suggested that ABA pre-treatment delayed the death of these cells when compared with the tissues in untreated nonviable buds that died after 24h of rehydration (Fig. 3.6F). Twenty excised buds were used per experiment (n=20), and each experiment was repeated three times. Scale bar: 1cm represents 10µm.


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Together with the viability studies, the histological and ultrastructural studies have furthered the understanding of the responses of *E. grandis* axillary buds to drying. The results demonstrated both the intolerance of the buds to excessive water loss and the beneficial effects of ABA. The drying process is a fundamental component of cryopreservation strategies (Uchendu and Reed, 2008, 2009). However, the removal of water from cells resulted in a number of stresses that were associated with the multiple roles that water played in supporting the life of the axillary buds. Stressful external processes can trigger both necrosis (the rapid death of the leaf primordia of the axillary buds) and programmed cell death (PCD) (the slower death of the meristematic tissues) (Locato *et al.,* 2008). In the latter, the biochemical and morphological events leading to death are usually organized in a cascade of very specific and controlled steps whereas necrosis is a non-physiological process that follows an overwhelming stress where the cells are unable to activate their apoptotic pathways (Reape *et al.,* 2008). The process of apoptosis is usually slower than necrosis occurring within a few hours or days, depending on the inducer (Lee *et al.,* 2009; Willingham, 1999).

Even though ABA provided some tolerance to partial dehydration of the *E. grandis* axillary buds, it was proposed that the drying process triggered necrosis in areas of cellular differentiation of the buds such as the leaf primodia and PCD in the meristem (less or not differentiated). The mechanisms of protection that were triggered by the application of ABA to the buds were not determined in this study. Nevertheless, the observed increased viability of axillary buds treated with ABA suggested that *E. grandis* buds had the ability to activate one or more ABA-dependent cellular protective mechanisms which promoted some resistance to water loss. This was demonstrated by the fact that when dried for 20min, ABA pre-treated buds maintained significantly higher water content than untreated buds [0.45g.g-1 ± 0.02g.g-1(ABA pre-treated) and 0.33g.g-1 ± 0.014g.g-1 (untreated)]. This was further supported by some of the histological results, such as the accumulation of phenolic compounds in ABA pre-treated buds (Figure 2.3B). It was, also, shown that the drying process had triggered necrosis and, possibly, PCD in the meristematic cells.

CHAPTER 3: PROGRAMMED CELL DEATH IN PARTIALLY DRIED *Eucalyptus grandis in vitro* AXILLARY BUDS

3.1 INTRODUCTION

The principal participants of PCD in animal cells are cysteine proteases, named caspases (Lockshin and William, 1964; Cohen, 1997; Fuentes-Prior and Salvesen, 2004; Gao *et al*., 2008). Most animal cysteine proteases associated with PCD (apoptosis) belong to a group of conserved ***c***ysteinyl-***asp***artate enzymes which cleave very specific peptide substrates after aspatic acid (Asp) residues, and therefore have been termed caspases (Zhang *et al*., 2009; Yamazaki *et al*., 2009; Han *et al*., 2012). These proteases function in the activation of a cascade and are present in most cells, residing in the cytosol as a single chain proenzyme. The plasma membrane contains specific receptors that bind respective ligands and these can be used to activate caspases (Budihardjo *et al*., 1999; Fuentes-Prior and Salvesen, 2004). Among the discovered caspases, caspase-3 is one of the main participants and most important protease in the caspase-dependent apoptotic process in animal cells, and it indicates the induction of the caspase cascade (Bras *et al*., 2005). To date, no true caspases have been found in plants. Nonetheless, there is accumulating evidence suggesting the existence of caspase-like proteases in plants (Lord and Gunawardena, 2012). The evidence includes studies based on the inhibitory effects of caspase-specific inhibitors and/or the cleavage of caspase substrates during plant PCD (Bonneau *et al*., 2008; Han *et al*., 2012). The existence of caspase-like proteases in plant PCD has led to the identification of two broad groups of caspase-like proteases: cystein endopeptidases (Rojo *et al*., 2004) and serine endopeptidases (subtilisin-like proteases in *Avena sativa* described by Coffeen and Wolpert, 2004) (Piszczek and Gutman, 2007). Furthermore, another group of subtilisin-like proteases, named phytaspases, were established as the plant counterpart of animal caspases (Chichkova *et al*., 2010). Phytaspases and caspases are structurally unrelated proteolytic enzymes, however, they display similar cleavage specificity and function (Chichkova *et al*., 2012). Overall, there is a vast amount of evidence demonstrating the role for caspase-like proteases in plant PCD.

During dehydration, plant tissue is subjected to a number of stresses. The type of damage that occur changes as the water content decrease and as the intensity of the stress increases. The lipid bilayer of the plasma membrane is known to undergo phase transitions during dehydration and rehydration (Caramelo and Iusem, 2009). Increased plasma membrane permeability during rehydration has been proposed as the main cause of cell death (Assani *et al*., 2009). The first indication that there is an alteration in cellular structure as a result of drying is the leakage of nucleotides, ions and other soluble cell components into external medium during rehydration of dried plant cells (Quartacci *et al*., 2002; Assani *et al*., 2009) . Such leakage of solutes from rehydrated cells is the result of an increase in permeability that occurs as the lipid bilayers of the membranes pass through a transition phase that is driven by the hydration state of the membrane (Quartacci *et al*., 2002).

3.2 MATERIALS AND METHODS

The maintenance of the parent plant material, the abscisic acid pre-treatment, the water content, viability, drying and re-imbibition of *in vitro* material were conducted as previously described (section 2.2.1 – 2.2.4 pages X - Y).

3.2.1 Measurement of extracellular superoxide production

Extracellular superoxide production was determined by using a colorimetric assay that measures the oxidation of epinephrine to adrenochrome spectrophotometrically at 490 mm (Misra and Fridovich, 1972). Hydrated shoot-clusters with 20 buds (Figure 3.1 A) each were placed onto acclimation medium (control clusters) for 7 days and abscisic acid ($5mg.l^{-1}$) supplemented medium for 5 days. Excised buds (Figure 3.1B) (hydrated, dried and rehydrated for 24h) and hydrated shoot-clusters were incubated in 4ml of 1mM epinephrine solution (pH7) and shaken at 45 revolution min^{-1} on a rotary shaker in the dark for 15min. A control was included which comprised the epinephrine solution without plant material. Superoxide production was calculated by using the molar extinction coefficient for adrenochrome (4020 l/mol/cm). Each experiment in the ABA pre-treatment and controls was replicated three times and included one hydrated shoot-cluster with 20 buds (n=1) and 20 excised buds (n=20, in each test for hydrated, dried and rehydrated buds).

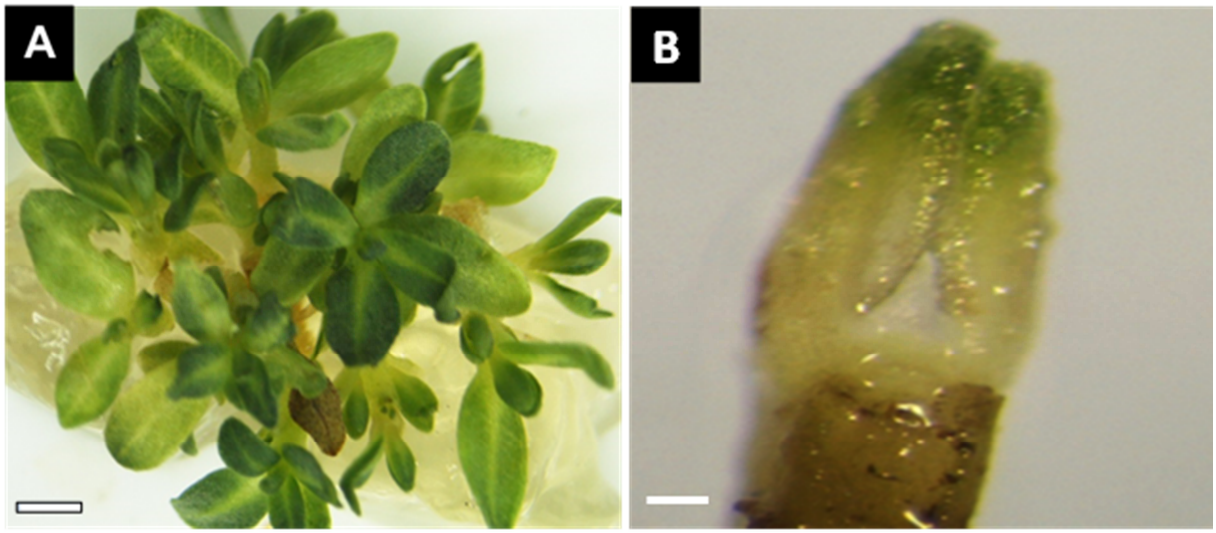


Figure 3.1. *E. grandis in vitro* shoot cluster (A) and isolated axillary bud (B). Eight week old shoot clusters and excised buds were used to determine the extracellular superoxide production in both ABA pre-treated and untreated plant material. Scale bar: 1cm represents 1.6mm (A) and 0.4mm (B).

3.2.2 Caspase assays

The procedure for the caspase assays was derived from Del Pozo and Lam (1998) and Mlejnek and Procházka (2002). Assays were performed on untreated and ABA pre-treated buds that were hydrated, dried (20min) and rehydrated over a period of 24 to 168 hours (episodically). Buds were placed in 30ml glass tubes and homogenized with an ice-cold extraction buffer [50mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 1mM ethylenediaminetetraacetic acid (EDTA), 1mM sucrose, 1mM dithiothreitol (DTT), 1mM phenylmethanesulphonyl fluoride (PMSF), 40mM 2-mercaptoethanol, 20% glycerol and 0.1% triton]. The homogenate was centrifuged (5000rpm) for 15min at 4ºC and the supernatant was removed using a micropipette for assessment. Protein extract (1ml per experiment) was obtained from 60mg in total mass of buds (± 120 buds). Each experiment was replicated three times.

3.2.2.1 Detection of caspase-3-like activity and inhibition

The reaction in 1ml of the protein extract (each from hydrated, dried and rehydrated buds that were untreated and ABA pre-treated) was initiated by adding 1ml of caspase-3 substrate (100µm Ac-DEVD-AMC). Caspase-3 inhibition was also measured by adding 1ml of caspase-3-specific inhibitor (300µm Ac-DEVD-CHO). A combination of protease inhibitors (300µm Ac-DEVD-CHO, 3mM pepstatin and 3mM leupeptin) was also used in the same manner.

All the samples were incubated for 1h at 30ºC. The reaction was stopped by adding 10µl of 36% (w/v) HCl. Samples were transferred into 4.5ml cuvettes and fluorescence was measured by using a Hoefer^{TM} DQ300 fluorometer at the excitation and emission wavelengths of 360 and 460nm, respectively. Protease assays were performed before and after 20min of drying, and over a period of 24 to 168 hours of rehydration (episodically).

3.2.3 Data and Statistical analysis

Caspase-3-like protease activity and inhibition data are presented in relative fluorescence units and are the mean ± SE of three replicates. Data was analysed with Statistica-version10: general linear model followed by a multiple comparison using Post-hoc Tukey Honest significant difference test where $\alpha = 0.05$.

3.3 RESULTS AND DISCUSSION

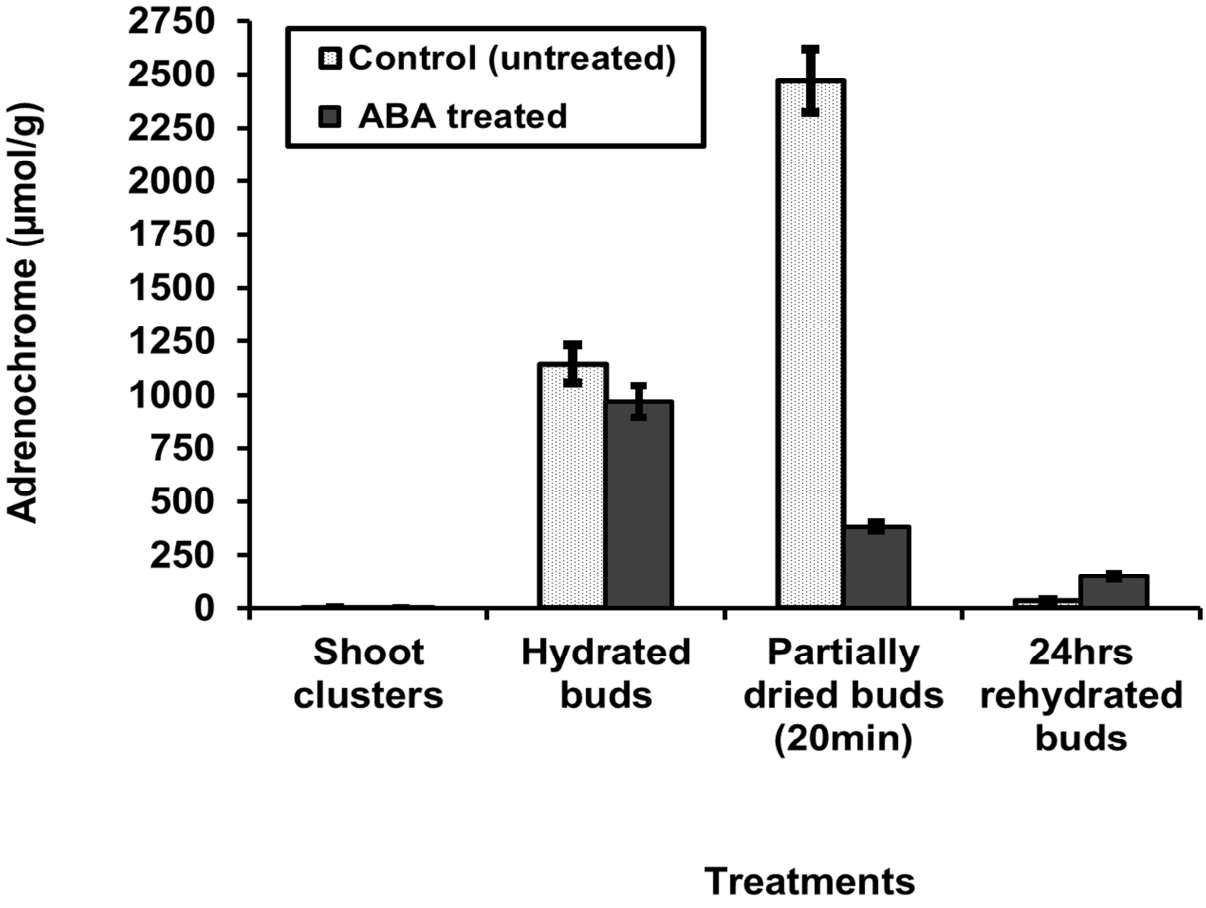
As previously mentioned, *E. grandis* axillary buds isolated from *in vitro* shoots were dried over silica gel for 20 minutes. Pre-treatment of the shoots with 5mg.l^{-1} ABA for 5 days resulted in partial resistance of the isolated buds to water loss (0.76g.g^{-1} ± 0.02g.g^{-1} to 0.45g.g^{-1} ± 0.02g.g^{-1} fresh mass basis) as compared with untreated (control) buds (0.76g.g^{-1} ± 0.02g.g^{-1} to 0.33g.g^{-1} ± 0.01g.g^{-1} fresh mass basis). Concomitantly viability decreased from 100 to 70% for ABA pre-treated buds and to 55% for the untreated buds. This approach, however, resulted in a number of stresses that triggered cell death processes. Various cell death triggers such as heat shock (Li *et al*., 2012), viruses (Solovieva *et al*., 2013), oxidative stress (Martínez-Fábregas *et al*., 2013) dehydration (Panis and Lambardi, 2006; Wright *et al*., 2009) and freezing can induce PCD and/or necrosis.

An abnormal increase in ROS production due to wounding, biotic or abiotic stresses may cause lethal damage (Roy *et al*., 2008; Whitaker *et al.,* 2010; Mittler *et al*., 2011; Serrano *et al*., 2012). Extracellular superoxide ($^{\cdot}O_2^-$) production in untreated clusters (0.395µmol/g ±0.010µmol/g) was lower than that in the ABA treated clusters [0.467µmol/g ±0.137µmol/g ($p<0.05$)] (Figure 3.2). It is probable that the $^{\cdot}O_2^-$ levels in untreated clusters were indicative of the normal production of ROS during physiological processes. Furthermore, the increase of ROS in ABA treated clusters was probably caused by ABA pre-treatment. ABA has been shown to increase ROS levels in plant material, for example in the leaves of bean seedlings (Mahmoodzadeh and Esparham, 2011) and *Arabidopsis* cell suspension cultures (Böhmer and Schroeder, 2011). Immediately after excision of the buds from the ABA pre-treated and untreated clusters there was a significant increase in the ROS levels [$F(1.8) = 162.89$, $p = 0.00$]. The increase in $^{\cdot}O_2^-$ production in those buds was presumably a stress response to the excision injury. This was further exacerbated by the 20 minutes of drying (Figure 3.2).

This was particularly apparent in material not pre-treated with ABA and these high ROS levels are considered to be the cause of the necrotic death of the majority of this material (Figure 2.6 B and F, chapter 2). This pattern of $\cdot O_2^-$ production was also observed with the isolation and drying of desiccation-sensitive axes of *Castanea sativa* (Roach *et al*., 2008), *Antiaris toxicaria* (Cheng and Song, 2008), *Trichilia.*

dregeana (Whitaker *et al*., 2010), *Strychnos gerrardii* (Berjak *et al*., 2011) and *Boophane disticha* (Berjak *et al*., 2011). Collectively, these data further support the role that ABA pre-treatment plays in preparing the material for the drying stress. In this regard it is possible that the observed accumulation of phenolics in the ABA pre-treated material (Figure 2.3B, chapter 2) played a part in the amelioration of the ROS (Figure 3.2). After 24h rehydration of the dried buds on medium the O_2^- level recorded in both untreated and treated buds had dropped but remained significantly higher than the apparent normal physiological levels [F (1.8) = 48.934, p = 0.00]. The $^{\cdot}O_2^-$ production after rehydration in the ABA pre-treated material was significantly higher than that in the rehydrated untreated buds, and this was interpreted as being linked to a probable rehydration stress in viable buds (70% ± 0.04%) and intact meristematic cells in non-viable buds (30% ± 1.55%) that eventually died after 96h (Figure 3.2).

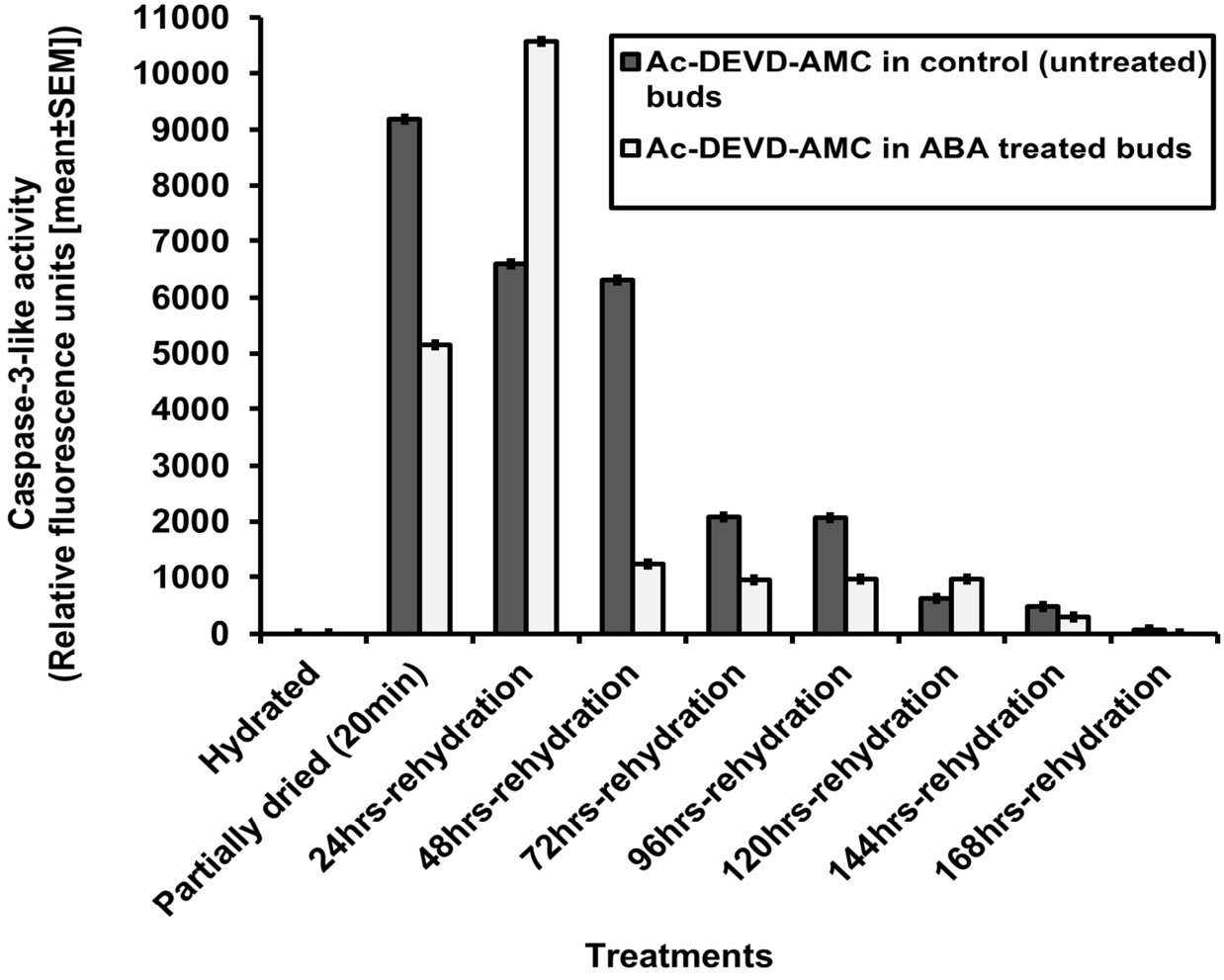
Figure 3.2. Production of extracellular superoxide in the control (untreated) and ABA pre-treated *E. grandis in vitro* clusters and axillary buds. The $^{\cdot}O_2^-$ production in untreated clusters (0.395µmol/g ±0.010µmol/g) was lower than that in the pre-treated clusters (0.467µmol/g ±0.137µmol/g) ($p<0.05$). The $^{\cdot}O_2^-$ levels in untreated clusters were probably indicative of the normal production of ROS during physiological processes. The increase of ROS in pre-treated clusters was probably caused by ABA pre-treatment. The significant increase [F (1.8) = 162.89, p = 0.00] in $^{\cdot}O_2^-$ production immediately after excision in both hydrated ABA pre- treated and control buds was probably reflecting a stress response to the excision injury. This was further exacerbated by the 20 min drying. After rehydration (24h), ROS production dropped sharply but remained significantly higher than the apparent normal physiological levels [F (1.8) = 48.934, p = 0.00]. The production in rehydrated pre-treated buds was interpreted as being linked to a probable rehydration stress in viable buds (70% ± 0.04%) and intact meristematic cells in non-viable buds (30% ± 1.55%) that eventually died. Each shoot cluster with 20 buds still attached (n=1) and 20 excised buds (n=20) were used. Each treatment was replicated three times.


Adrenochrome (µmol/g)
2750
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
Control (untreated)
ABA treated
Shoot clusters
Hydrated buds
Partially dried buds (20min)
24hrs rehydrated buds
Treatments

Mitochondria are important sources of ROS, which are thought to act as signals during plant PCD regulation (Navrot *et al*., 2007; Zsigmond *et al*., 2008; Roy *et al*., 2008; Doyle *et al*., 2010; He *et al*., 2012). An increased production of ROS due to biotic or abiotic stresses (such as artificial drying) may result in a release of cytochrome *c* from the mitochondria into the cytosol and a subsequent activation of caspase-like proteases, which have been shown to be involved in the control of death activation in plants (Roy *et al*., 2008; Roach *et al*., 2010; Serrano *et al*., 2012). The observed changes in the mitochondrial profiles of intact cells of the meristematic domes of dried axillary buds could indicate that the drying process resulted in stresses and/or disruptions of the ATP generation processes which lead to more production of ROS and subsequently activated the death process. Caspase-3-like protease activity may have been triggered in such a manner during the drying process of both the untreated and the ABA pre-treated nonviable buds. In this respect the caspase-3-like activity in the untreated buds was significantly higher than in the ABA treated buds [$F(2, 18) = 5358 \times 10^4$, $p<0.05$]. At that same time, the highest ROS production was observed in untreated buds. This suggested that the cryopreparative drying triggered the activation of caspase-3-like protease in the nonviable untreated and treated buds.

An increase in ROS production that was accompanied by an increase in caspase-like activity was also observed in material such as microspores and cell suspension cultures (of barley) after a stress treatment (Rodríguez-Serrano *et al*., 2012). Notably, the process of isolating the *E. grandis* buds, which involved excision of buds from shoot clusters, did not activate the caspase-3-like protease as a fluorescent signal was not detected in the hydrated controls.The highest activity was observed after 24h rehydration on acclimation medium in ABA pre-treated buds followed by a sharp decrease in activity after 48 hours of rehydration (Figure 3.3).

Figure 3.3. Detection of caspase-3-like protease activity in the control (untreated) and ABA pre-treated *E. grandis in vitro* axillary buds. Caspase-3-like protease was activated during the drying process in both untreated and pre-treated nonviable buds. The process of isolating buds which involved excision of buds from shoot clusters did not activate the caspase-3-like protease as a fluorescent signal was not detected in both untreated and pre-treated the hydrated buds. A high caspase-3-like activity was observed in untreated buds after partial drying (20min in silica gel) as compared with pre-treated dried buds [F (2.18) = 5358 x10^4, p<0.05].The highest activity was observed after 24 hours of rehydration on acclimation medium in pre-treated buds followed by a sharp decrease in activity after 48 hours of rehydration [F (2.18) = 5358 x10^4, p<0.05]. A decrease in activity after 72 hours of rehydration was observed in both ABA pre-treated and control buds. Therefore, this was indicative of cell death. Data are presented in relative fluorescence units (mean ± SE), and are the mean of three separate experiments.

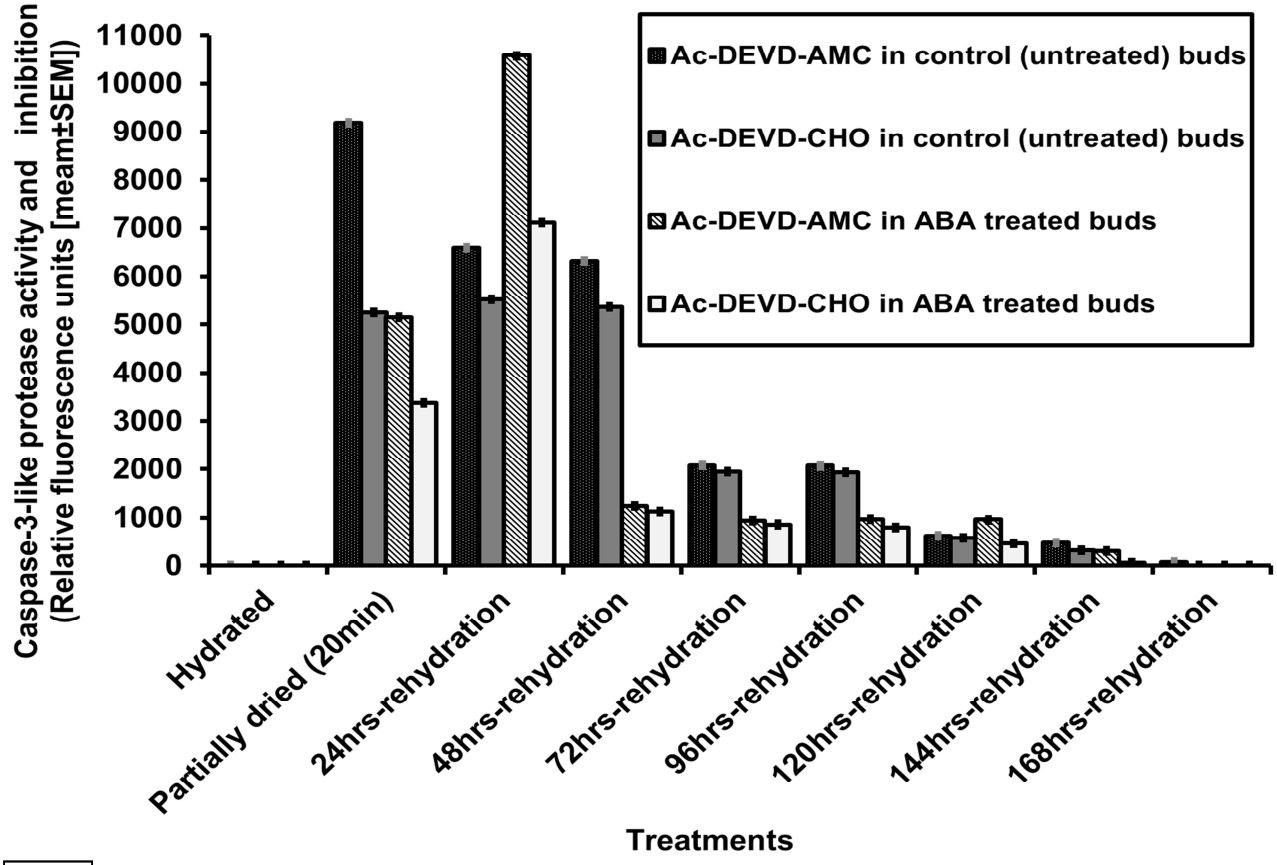

Caspase-3-like activity
(Relative fluorescence units [mean±SEM])
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Ac-DEVD-AMC in control (untreated) buds
Ac-DEVD-AMC in ABA treated buds
Hydrated
Partially dried (20min)
24hrs-rehydration
48hrs-rehydration
72hrs-rehydration
96hrs-rehydration
120hrs-rehydration
144hrs-rehydration
168hrs-rehydration
Treatments

Rehydration is not a simple recovery process and in itself is also a stressful process (Caramelo and Iusem, 2009).The lipid bilayer of the plasma membrane is known to undergo phase transitions during dehydration and rehydration (Caramelo and Iusem, 2009). Indeed increased plasma membrane permeability during rehydration has been proposed as the main cause of cell death (Higo *et al*., 2008). The high caspase-3-like activity observed after 24h of rehydration in ABA pre-treated buds was attributed to an added rehydration stress which further stimulated its activity. A decrease in activity after 72 hours of rehydration in both untreated and ABA pre-treated nonviable buds was indicative of cell death. Caspase-like activity in plants has been detected in cases associated with severe stresses, such as heat shock (Tian *et al*., 2000), treatment with toxic chemicals (De Jong *et al*., 2000; Jiménez *et al*., 2009) and pathogen reaction (Del Pozo and Lam, 1998; Li *et al*., 2008; Serrano *et al*., 2012). Futhermore, caspase-like activity was reported for several stress-triggered PCD processes, such as hypersensitive reactions and chemical treatments (Chichkova *et al*., 2004). It seems, therefore, in nonviable treated buds the stresses associated with the cryopreparative drying and subsequent rehydration probably caused an activation and a further increase of caspase-3-like activity after 24h rehydration which was followed by a sharp decrease in activity.

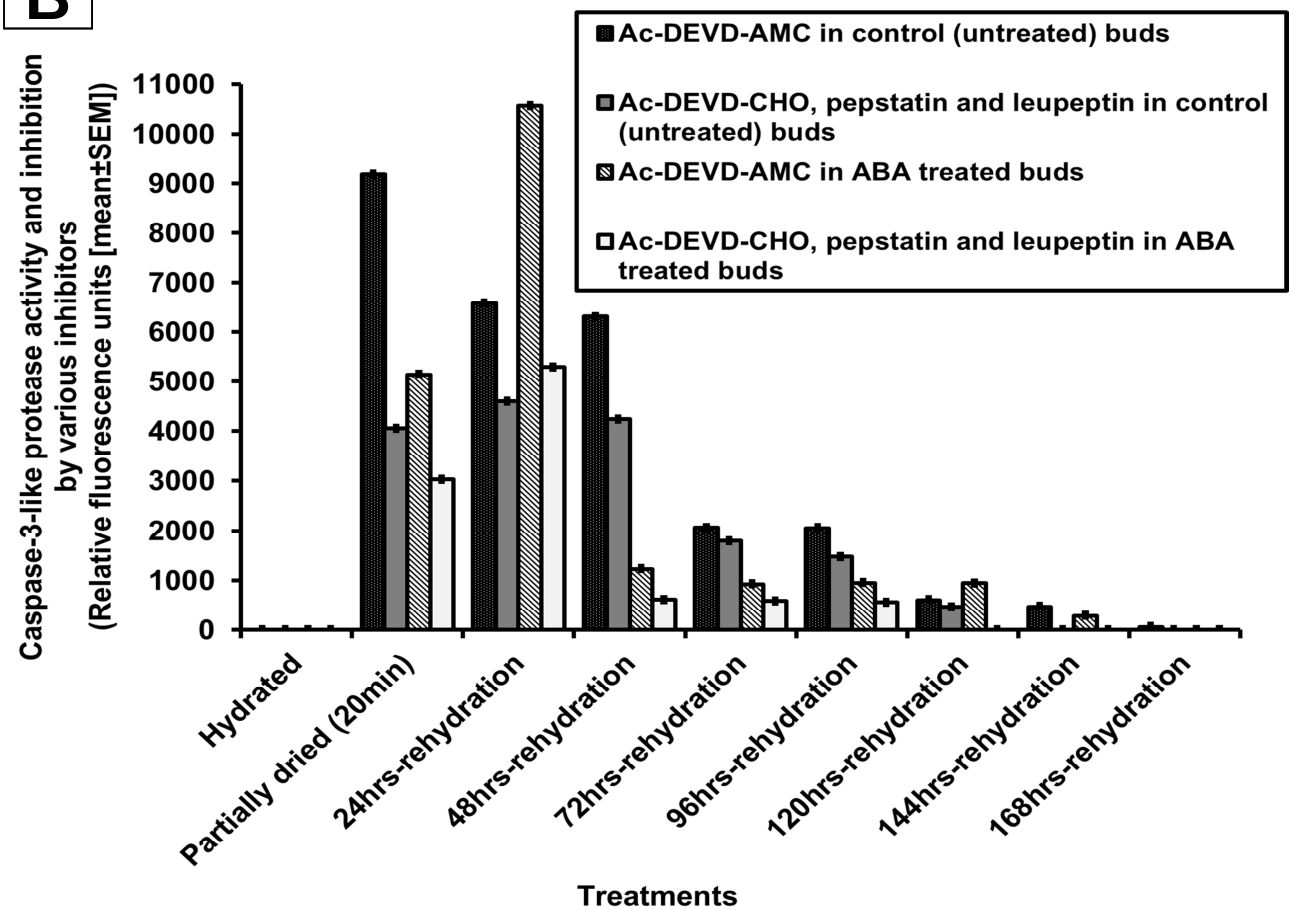
The involvement of caspase-3-like activity in the execution of PCD in the *E. grandis* axillary buds was further supported by the inhibitor studies (Figure 3.4A-B). An increasing number of reports show that inhibitors to various mammalian caspases markedly suppress plant cell death (e.g. Bosch and Franklin-Tong 2007; Zhang *et al*., 2009; Rodríguez-Serrano *et al*., 2012). The selective and reversible synthetic tetrapeptide inhibitor of Caspase-3, Ac-DEVD-CHO, partially suppressed the caspase-3-like activity (Figure 3.4A). The addition of the other inhibitors pepstatin and leupeptin suppressed the activity even further especially after drying in the untreated buds [$F(1, 12)=2287 \times 10^4$, $p<0.05$] and after 24h rehydration in ABA treated buds [$F(1, 12)=1399 \times 10^4$, $p<0.05$] (Figure 3.4B). Collectively these data indicated that PCD was triggered in the axillary buds. This strongly supports the suggestion that activation of caspase-like proteases involved in many plant systems undergoing PCD might be abolished by caspase inhibitors (Lam and del Pozo, 2000; Rodríguez-Serrano *et al*., 2012).

Figure 3.4. The effect of caspase-3-specific selective peptide inhibitor (A) and various protease inhibitors (B) pepstatin, leupeptin and acetyl-Asp-Glu-Val-Asp-aldehyde on caspase-3-like protease activity. This inhibitor partially suppressed caspase-3-like activity after drying (20min) and rehydration (24 to 168h episodically) in both ABA pre-treated and control buds (A). The addition of pepstatin and leupeptin (B) suppressed the activity even further especially after drying (control buds) [F (1.12) = 2282 x10^4, p<0.05] and after 24h rehydration (ABA pre-treated) [F (1.12) = 1399 x10^4, p<0.05]. The inhibitor sensitivity study showed that although caspase-3 specific inhibitor could not completely suppress the caspase-3-like activity (A) when it was used without additional inhibitors, the activity was markedly suppressed (B) when all three inhibitors (300µm Ac-DEVD-CHO, 3mM pepstatin and 3mM leupeptin) were combined (p<0.05). Data are presented in relative fluorescence units (mean ± SE), and are the mean of three separate experiments.

A


Caspase-3-like protease activity and inhibition (Relative fluorescence units [meam±SEM])
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Ac-DEVD-AMC in control (untreated) buds
Ac-DEVD-CHO in control (untreated) buds
Ac-DEVD-AMC in ABA treated buds
Ac-DEVD-CHO in ABA treated buds
Hydrated
Partially dried (20min)
24hrs-rehydration
48hrs-rehydration
72hrs-rehydration
96hrs-rehydration
120hrs-rehydration
144hrs-rehydration
168hrs-rehydration
Treatments

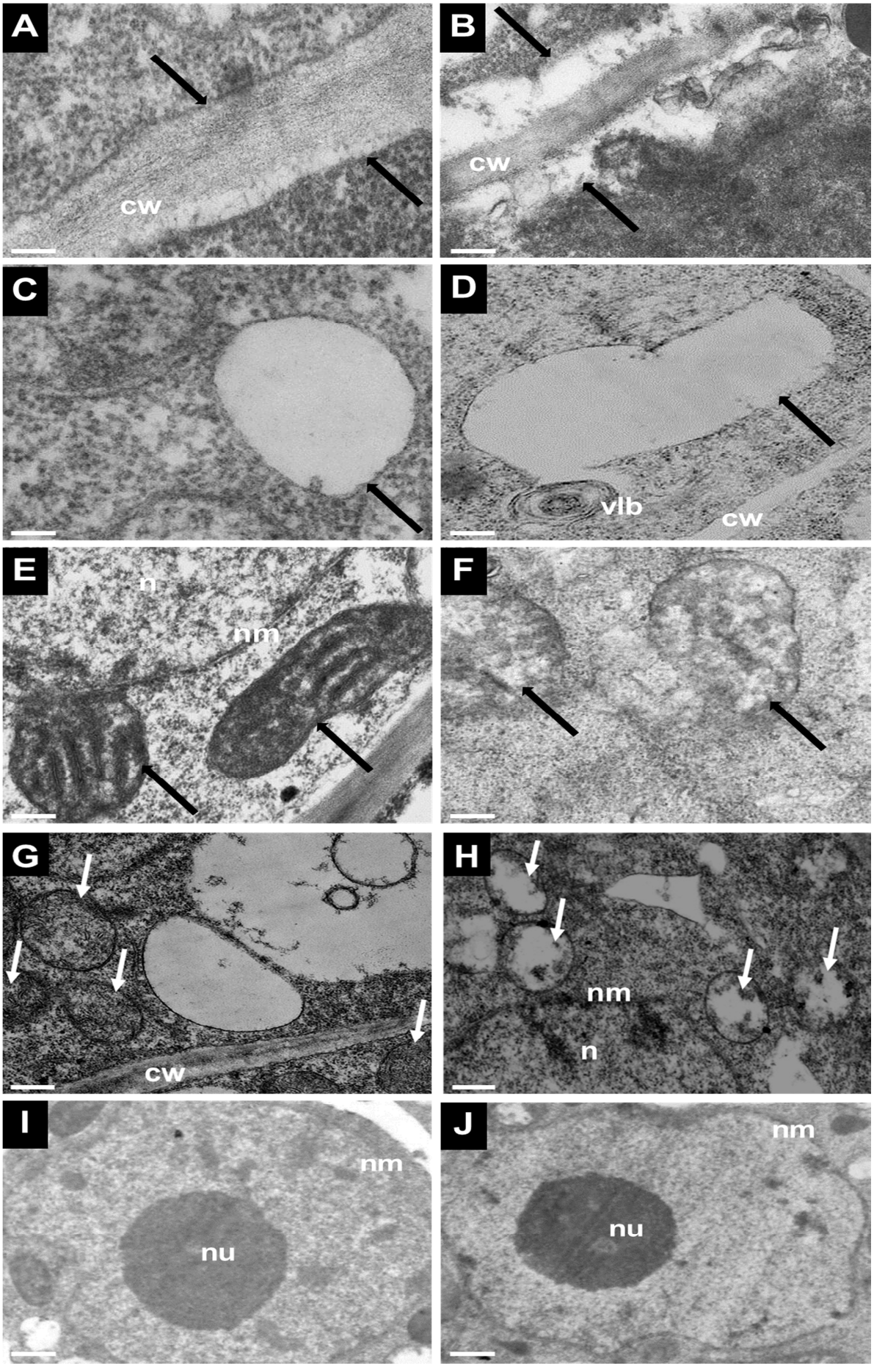
B


Caspase-3-like protease activity and inhibition by various inhibitors (Relative fluorescence units [mean±SEM])
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Ac-DEVD-AMC in control (untreated) buds
Ac-DEVD-CHO, pepstatin and leupeptin in control (untreated) buds
Ac-DEVD-AMC in ABA treated buds
Ac-DEVD-CHO, pepstatin and leupeptin in ABA treated buds
Hydrated
Partially dried (20min)
24hrs-rehydration
48hrs-rehydration
72hrs-rehydration
96hrs-rehydration
120hrs-rehydration
144hrs-rehydration
168hrs-rehydration
Treatments

As previously mentioned, there are proteases that are responsible for the caspase-like activities in plants (Coffeen and Wolpert, 2004; Bozhkov *et al*., 2005; Bozhkov *et al*., 2010). Furthermore, the identification of plant analogues of caspases was initiated based on caspase-3, a major human cell death executioner, capable of inducing cleavage of the VirD2 protein encoded by the plant pathogenic bacterium *Agrobacterium tumefaciens* (Chichkova *et al*., 2004). Plant protease capable of cleaving VirD2 in a caspase-3-like manner was indeed detected. Hence, a subtilisin-like protease as a counterpart of animal caspases, phytaspase, was established (Chichkova *et al*., 2012). Therefore, the present study suggests that different plant proteases might have been active during PCD in nonviable ABA pre-treated buds; proteases that have similar substrate and inhibitor properties as the mammalian caspase3. Meristematic cells in ABA pre-treated non-viable rehydrated (24h) buds exhibited various PCD features. The plasma membrane was withdrawn from the cell wall (Figure 3.5B), cell walls were thin and disrupted (Figure 3.5B), the tonoplast had ruptured (Figure 3.5D), elaborate multi-membraned vescicle-like structures had formed (Figure 3.5D), mitochondrial internal membranes were degraded (Figure 3.5H) and intact nuclei were characterized by chromatin clumping (Figure 3.5J). Those features have been described in other cases of plant PCD. For instance, with progression through the PCD process in *Aponogeton madagascariensis*, the separation of the plasma membrane from the cell wall, tonoplast rupture, chromatin condensation, appearance of vesicle structures, thinned cell walls (Wertman *et al*., 2012) and degraded mitochondrial cristae (Lord *et al*., 2011). In meristematic cells, the development of chloroplasts from proplastids is often relatively direct. Thus chloroplasts are commonly regarded as an end product of plastid development (Bräutigam *et al*., 2009). During plant PCD, the chloroplast matrix and internal membrane structure is degraded. This feature was observed in other plant systems such as senescing leaves and cells (Zapata *et al*., 2005; Rosenvasser *et al*., 2006; Hörtensteiner and Lee, 2007), and lace plant cells (Wright *et al*., 2009; Lord and Gunawardena, 2011). Interestingly, the proplastid membrane degradation was observed in ABA pre-treated *E. grandis* meristematic cells undergoing PCD (Figure 3.5F). Although the proplastids in those cells were undifferentiated, the responses were similar to those observed in membranes of chloroplasts in plant cells which have undergone PCD (Lord and Gunawardena, 2011).

It is generally accepted that multiple forms of plant PCD exist and that some of them do not require caspase-like activation (Smetana *et al*., 2012). However, there is increasing evidence regarding PCD that is induced by biotic and abiotic stresses that result in the generation of ROS and, presumably, activation of the caspase-like proteases (Reape and McCabe, 2010; Rodríguez-Serrano *et al*., 2012). Data in the present study showed the role of both necrosis and PCD in the death of the tissues of the axillary buds of *E. grandis* axillary buds. The data also contributed to the better understanding of the impact of cryoprocedures on these clonal tissues which are ideal propagules for forestry germplasm conservation.

Figure 3.5. Comparison of the subcellular features (arrows) of the meristem cells found in ABA pre-treated *Eucalyptus grandis* hydrated buds and buds rehydrated for 24h. Organelles in hydrated cells had intact plasma membranes pressed against the cell walls (A), intact vacuoles and tonoplasts (C), proplastids (E), mitochondria with intact cristae (G) and the nucleus (I). In comparison, cells undergoing PCD exhibited plasma membrane retraction from the cell wall(B), tonoplasts rupture (D), vesicle-like bodies near damaged vacuoles (D), degraded proplastids with less clear inner and outer membranes, mitochondrial inner membrane clearing (H), and intact nuclei characterized by chromatin clumping (J). Scale bar: 1cm represents 0.28µm (A), 0.33µm (B), 0.31µm (C), 0.38 µm (D), 0.36µm (E), 0.36 µm (F), 0.5 µm (G and H) and 0.6 µm (I and J).


A
cw
B
cw
C
D
vlb
cw
E
n
nm
F
G
cw
H
nm
n
I
nm
nu
J
nm
nu

CHAPTER 4: GENERAL DISCUSSION, FUTURE RECOMMENDATIONS AND CONCLUDING REMARKS

Long-term storage of clonally propagated plant species of commercial value is a critical component of plant breeding programmes (Reed *et al*., 2012). However, in South Africa land for field-base clonal maintenance is becoming increasingly scare. Therefore, the development of alternate methods of maintaining such material is essential (Mycock *et al*., 2004). Cryopreservation has proven to be an important and valuable method for the long-term preservation of biological material (Benson, 2008). The advantages of cryopreservation lie in the fact that germplasm can be effectively preserved for extended periods with low costs and with little space utilisation. Rapid progress in cryopreservation has been made and different plant materials such as cells, tissues and organs can be cryopreserved (Reed *et al*., 2012). Cryobiological studies of plant materials have established numerous protocols and generally these are specific to a particular species (Burritt, 2008). However, irrespective of the protocol used each step in a cryopreservation protocol has the potential to impose a stress on the plant material and when combined these can prove to be lethal (Reed *et al*., 2012). Therefore, the success of a cryo-protocol depends on the tolerance and sensitivity of the plant material to such stresses (Kaviani, 2011).

There are two important concerns: 1) ability to regenerate and multiply explants after retrieval from liquid nitrogen; 2) the maintenance of the genetic integrity of the cryopreserved material, which is particularly important in clonal forestry. Axillary buds meet these criteria because of the potential to regenerate a large number of explants from an individual bud and the minimal risk of somaclonal variation (Engelmann, 2000; Reed, 2001).This criterion is an absolute requisite for the storage of clonally propagated, highly selected, superior genotypes of *Eucalyptus*. The choice of explants selected for storage is very important as the occurrence of somaclonal variation is a threat for such material especially if regeneration occurs indirectly (e.g. through callus) (Engelmann, 2000). In this regard, *in vitro* axillary buds cultures are usually considered the most suitable because of their inherent genetic stability (Takagi, 2000). In addition, *in vitro* buds were selected because direct bud multiplication protocols have been established for most of the economically important

eucalypt species and hybrids, and are routinely used (Donald and Newton, 1991; Le Roux and van Staden, 1991; Watt *et al*., 2003; Jones and van Staden, 1994).

Despite their suitability as the explants of choice for cryostorage, to date a successful protocol for cryostorage of *Eucalyptus* buds has not been achieved because of their desiccation (Padayachee *et al*., 2009). This presumably being a reflection of their tropical/ sub-tropical origin, hence they are highly hydrated (Christie, 2008; Grattapaglia, 2008).

The ultimate aim in any cryo-protocol is the avoidance of ice crystals formation which if formed are usually lethal. Plant material must be dried to avoid the formation of ice crystals (Engelmann, 2012) and cryo-protocols generally make use of chemical and physical methods to dehydrate the cryo-propagule. Sensitivity to the process of drying varies among species (Rajasekharan, 2006). *Eucalyptus grandis* is sensitive to excessive water loss. This limitation was clearly demonstrated by the loss of both vigour and viability of the *in vitro* control buds that were dried and then rehydrated. The sensitivity to drying *in vitro* plant material may be overcome by inducing tolerance to the drying. The application of exogenous ABA has been associated with the activation of stress response pathways (Fang *et al*., 2004). Several authors have described the effect of ABA in the preculture medium on stress tolerance in various species and found that ABA significantly improves the recovery of cryopreserved explants (reviewed by Rai *et al*., 2011). With respect to optimising the drying step in the cryo-procedure for *E. grandis in vitro* axillary buds, it has been established that the exogenous application of 5mg.l^{-1} ABA induces some resistance to water loss (Padayachee *et al*., 2008). This was indicated by the fact that when dried for 20min over silica gel ABA pre-treated buds maintained significantly higher water content than dried control buds [0.45g.g^{-1} ± 0.02 g.g^{-1} (ABA pre-treated buds) and 0.33g.g^{-1} ± 0.014 g.g^{-1} (control buds)]. Those findings and those presently obtained suggest that *E. grandis* buds have the ability to activate one or more ABA-dependent stress tolerance mechanisms which promote some resistance to water loss. Further evidence supporting the latter was the accumulation of phenolics in ABA pre-treated buds (Figure 2.3B, chapter 2).

The viability of ABA pre-treated buds, however, was negatively impacted by drying decreasing from 100% ± 0% to 70% ± 0.04% (Figure 2.2, chapter 2). Consequently,

it was hypothesized that the drying process may have triggered necrosis or programmed cell death (PCD) in axillary buds. The collected data suggest that the non-viable buds had undergone necrosis (control buds and differentiated tissues in ABA pre-treated buds) and PCD in the meristem cells (ABA pre-treated buds). This was demonstrated by the fact that after 24h of rehydration the ultrastructure of necrotic cells showed extensive damage or complete loss of cellular integrity (Figure 2.5A and 2.5C), whereas the meristem cells in the ABA pre-treated buds exhibited a mosaic pattern in cellular integrity (Figure 2.5B and 2.5D). The viability studies further confirmed and supplemented the LM and TEM results (Figure 2.7). In addition, the PI stain results (obtained after 24, 48 and 72h rehydration) substantiated that the meristem cells in ABA in ABA pre-treated non-viable buds had undergone the PCD process (Figures 2.6H, 2.7B and 2.7D), and illustrated that these eventually died after 96h rehydration (Figure 2.7F).

The isolation of the axillary buds from the shoot clusters resulted in a significant increase in $^{\cdot}O_2^-$ production. This was further exacerbated by the 20min drying in the control buds (Figure 3.2). As pointed out by Reed (2012), it is a well-established fact that the steps in cryo-protocols impose stresses that may increase ROS accumulation in plants. Nevertheless, the positive effect of ABA was apparent as it appeared to reduce the generation of $^{\cdot}O_2^-$ after subsequent drying. However, as stated by several authors (e.g. Nawkar *et al*., 2013; Tran *et al*., 2013) accumulation of ROS in various stresses has been shown to be involved in the initiation of plant PCD. Indeed, this study demonstrated that although ABA application may have been partially successful in inducing tolerance to stress, ROS accumulation was apparently not adequately reduced. Consequently, the PCD process was activated.

In the meristem cells that had undergone PCD, it is suggested that the mitochondria have played an important role in the PCD process. This was evidenced by the fact that the mitochondria were observed to have damaged internal membranes (Figure 3.5H). That observation, therefore, suggested that the excess ROS accumulation in those cells was mainly derived from the mitochondria. Furthermore, ROS may have further damaged other cellular structures such as proplastids, membrane lipids and proteins. Concomitantly, the mitochondrial dysfunction including the loss of mitochondrial transmembrane potential may have led to the opening of the

permeability transition pore, which led to the release of cytochrome *c*, and subsequent activation of caspase-like proteases.

The release of cytochrome *c* from the mitochondria has been suggested in various types of plant PCD (Martinez-Fábregas *et al*., 2013; Nawkar *et al*., 2013). Although, the cytochrome *c* release from mitochondria into the cytosol was not assessed in this study, the evidence of caspase-3-like protease activation suggests the existence of functional caspase-like proteolytic activity in the meristem cells that were undergoing PCD (Figure 4.1). Further support for this hypothesis was the partial inhibition of the caspase-3-like activity (Figure 3.4). In addition, the occurrence of the PCD process was further substantiated by the observed tonoplasts rupture, chromatin clumping, appearance vesicle-like structures, plasma membrane retraction from the cell walls and thinned cell walls (Figure 3.5).

Excessive accumulation of ROS is a common response of plant material to physical or biological stresses, and may cause irreversible damage that can lead to necrosis and/or PCD activation (Chowdhurya *et al*., 2008). As already discussed, among a variety of events occurring during PCD, the release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol is considered as a crucial regulatory step in PCD (Bobba *et al*., 2004). In various plant model systems, cytochrome *c* release has been shown to depend on the production of ROS, which could trigger PCD (Nawkar *et al*., 2013; Tran *et al*., 2013). In this study, increased ROS production due to drying was clearly confirmed. It is, therefore, recommended that investigations link ROS production and cytochrome *c* release from the mitochondria. This may be achieved by measuring the amount of cytochrome *c* in the cytosolic and mitochondrial fractions before and after drying. The analysis could be carried out by using immunoblotting (using a monoclonal antibody against cytochrome *c*) and polarographic techniques, as described by Vacca *et al*., (2006). In addition, to ascertain whether cytochrome *c* release is dependent on the production of ROS caused by drying, plant material may be analysed by using ROS scavenging enzymes such as superoxide dismutase and catalase (Vacca *et al*., 2006).

Another aspect that was considered was the effect of specific peptide inhibitors of caspases. It was demonstrated that, although the application of inhibitors such as pepstatin, leupeptin and acetyl-Asp-Glu-Val-Asp-aldehyde was partially successful,

caspase-3-like activity was not completely inhibited. It is important, therefore, to continue to determine the effect of inhibitors by introducing the combined inhibitors (pepstatin, leupeptin and acetyl-Asp-Glu-Val-Asp-aldehyde) prior to the drying process. This may be achieved by using the simple technique of incubating the material in solutions containing inhibitors (Vacca *et al*., 2006; Xu and Zhang, 2009; del Pozo, 1998). The addition of inhibitors before drying could potentially inhibit the activation of caspase-3-like protease and, ultimately, the PCD process.

High antioxidant status in plants material is associated with tolerance to cryopreservation methods (Burritt, 2008). Although not tested in the present study, the combined exogenous application of ABA and proline in the pre-treatment medium has been found to greatly increase the ROS scavenging capacity in, for example, *Begonia x erythrophylla* shoots (Burritt, 2008) and *Vicia faba* leaves (Ali *et al*., 2013). The response of *E. grandis in vitro* axillary buds to ABA demonstrated the potential for preconditioning the tissues to tolerate stresses associated with cryo-procedures such as partial drying (Ting *et al*., 2014). The addition of proline, if combined with ABA, could potentially reduce the ROS generation because proline is one of the important antioxidants proven to be a ROS scavenger and potential inhibitor of PCD (Ashraf and Foolad, 2007; Burritt 2008; Ali *et al*., 2012, 2013; Siddiqui *et al*., 2013).

The combined evidence from the present study has substantiated the hypothesis that the cryo-preparative drying step in the existing cryo-protocol triggered PCD and necrosis. The limitation (viability decreasing from 100% ± 0% to 70% ± 0.04%) that may be imposed by the sensitivity of *E. grandis in vitro* buds to the process of cryo-preparative drying were also clearly delineated. Encouragingly, however, the understanding of the impact of cryo-procedures on these clonal tissues has been improved

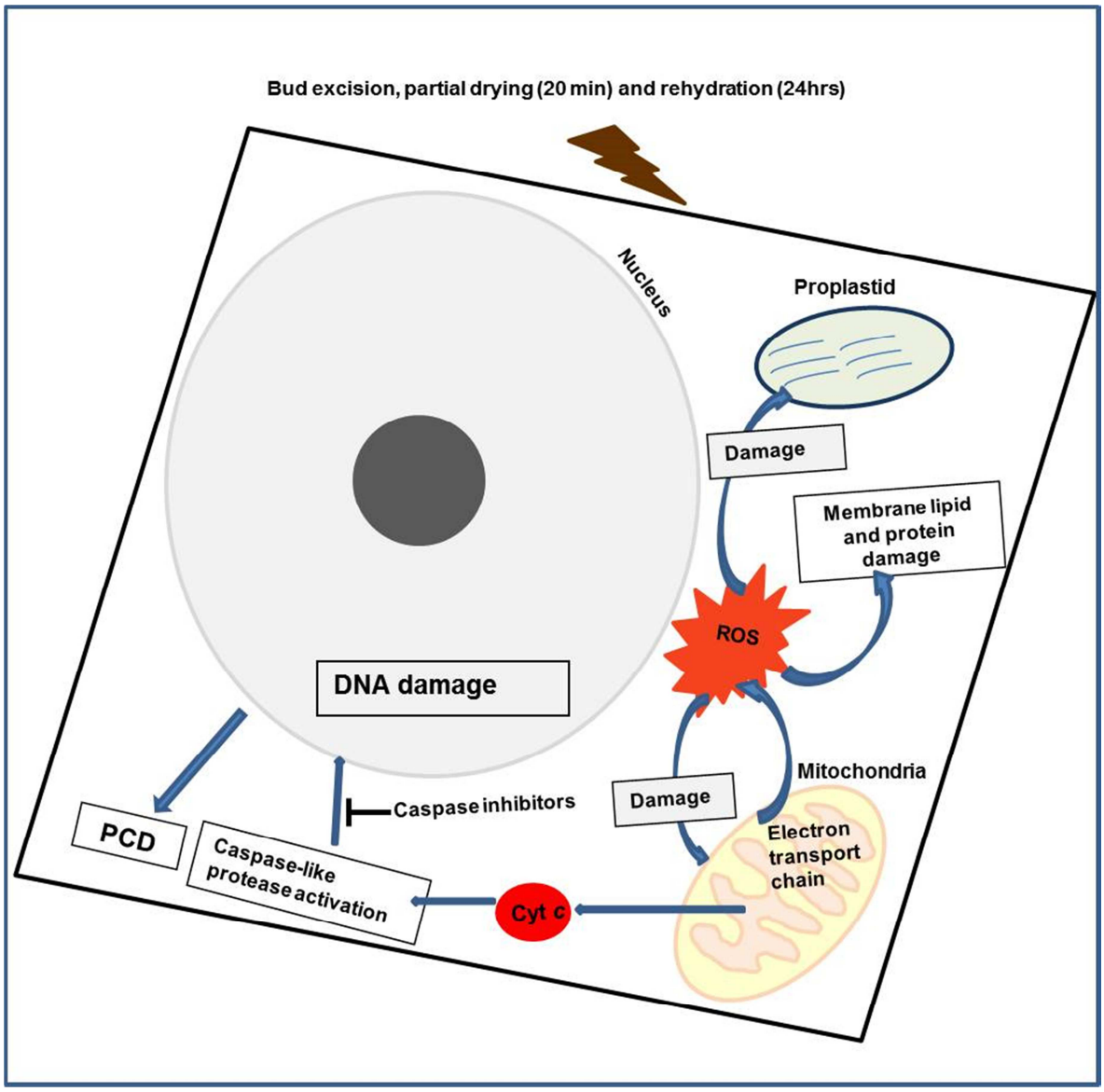


Figure 4.1. Schematic representation of a proposed pathway in a ABA pre-treated *in vitro* axillary bud meristematic cell of *E. grandis* undergoing programmed cell death. Bud excision injury, partial drying and rehydration results in an increased burst of ROS, which causes membrane lipid, protein and proplastid damage. Furthermore, ROS causes mitochondrial transmembrane potential loss which results in cytochrome *c* and activation of caspase-like cascade of events, which leads to PCD.

In conclusion, the novel information and, therefore, the contribution of this study include: 1) bud excision injury, partial drying and rehydration results in increased levels of ROS, which cause membrane lipid, protein and proplastid damage and 2) the elevated ROS causes the activation of caspase-3-like protease(s), which leads to PCD.

The understanding of the impact of cryo-procedures on these clonal tissues has been improved. The success of the cryo-protocol could have significance in commercial forestry, conservation of many clones and broadening the genetic base for plantation tree species.

APPENDIX: Transmission electron microscopy fixation and staining

1. Phosphate buffer

Solution A: 14.2g.l^{-1} disodium hydrogen orthophosphate anhydrous (Na_2HPO_4)

Soluion B: 15.6 g.l^{-1} Sodium dihydrogen orthophosphate dehydrate ($Na_2HPO_4.2H_2O$)

To make 100ml of buffer solution, mix 72ml of Solution A with 28ml of Solution B.

2. Glutaraldehyde Fixative (3% v/v)

Mix 2ml of 25% (v/v) glutaraldehyde stock solution with 88ml of phosphate buffer.

Keep the solution refrigerated. To fix specimens, cover with fixative solution and allow to stand for 2h to overnight.

3. Osmium tetroxide (OsO_4)

Mix one vial of OsO_4 with an equal volume of phosphate buffer in the fume cupboard. Cover the specimen with OsO_4/buffer mixture and leave in the fume cupboard for 1h.

4. Uranyl acetate (UA) (1% w/v)

Dissolve 0.25g of UA with 25ml of 75% (v/v) ethanol. Wrap the bottle with foil as solution is light sensitive. The solution must be made up fresh immediately prior to use

5. Epoxy resin embedding medium (Spurr, 1969)

Components:

- Resin: vinylcyclohexene dioxide (CVD)
- Hardener: nonelyl succinic anhydride (NSA)
- Plasticiser: DER 736
- Accelerator: di-methylaminoethanol (DMAE)

Components must be at room temperature for use.

Add components gravimetrically to a suitable container:

VCD 23g

NSA 62g

DER 736 14g

DMAE 1g (must be added after the other components have been added and mixed).

Standard polymerization time: 8 – 12h at 70ºC.

Fixing and embedding procedure:

- Place samples in gluteraldehyde fixative for the appropriate amount of time.
- Change to buffer, making one double change followed by five further changes over 1h.
- Replace buffer with OsO_4 and keep in fume cupboard for 1h.
- Make up UA.
- Remove OsO_4 and change to buffer. Change the buffer twice more over ½h.
- Change to 10% (v/v) ethanol then to 25% and 50% (v/v) ethanol after 15min in each solution.
- Replace 50% (v/v) ethanol with UA solution, wrap samples with foil and keep refrigerated for 1h.
- Remove UA solution and follow with two double changes of 75% (v/v) ethanol (10min each).
- Remove 75% (v/v) ethanol and replace with absolute ethanol.
- Two changes of absolute ethanol (10min each)
- Remove absolute ethanol and follow with two changes of propylene oxide (10min each)
- Add equal amounts of resin and propylene oxide to samples after second change of propylene oxide. Leave for 5h.
- Remove propylene oxide/resin mixture and replace with whole resin. Leave for overnight.
- Remove whole resin, place samples in resin trays. Fill with fresh resin and incubate in an oven at 70ºC for 8-12h.

Staining solutions:

1. Lead citrate

- Place 1.33g of lead citrate and 1.76g of sodium citrate ina 50ml flask with 30ml of distilled water.
- Shake vigorously for 1min.
- Allow to stand for 30min to 1h, shaking occasionally
- When the solution turns completely milky, add 8ml of freshly made 1N sodium hydroxide.
- Make up to 50ml with distilled water.
- Gently rotate if solution is still milky.
- Final solution should be clear.

2. Uranyl acetate

- Dissolve 0.25g of UA with 25ml of 75% (v/v) ethanol.
- Wrap the bottle with foil as solution is light sensitive.

Staining procedure:

- Place specimens on grids, in specimen holder.
- Using a clean syringe and filter, cover the specimens with lead citrate.
- Leave lead citrate to stain for 15min.
- Rinse thoroughly by the immersing specimen holder in distilled water.
- Blot-dry with filter paper and cover specimens with filtered UA.
- Leave UA to stain for 7 – 10min.
- Rinse thoroughly and blot-dry, and leave to dry completely prior to viewing.

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